European Commission

Combined Draft Renewal Assessment Report prepared according to Regulation (EC) N° 1107/2009
and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

Glyphosate

Volume 3 – B.9 (AS)
Ecotoxicology data
Appendix Literature data

Rapporteur Member State: Assessment Group on Glyphosate (AGG) consisting of FR, HU, NL and SE

Version History

When	What
2021/06	Initial RAR

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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APPENDIX TO VOL. 3 B.9 ECOTOXICOLOGY DATA

The strategy of the literature search on ecotoxicology data is summarised and presented under Volume 3 (AS) B.9., point B.9.11.1.

The list of publications excluded from the risk assessment after detailed assessment of full-text documents is presented in table B.9.11.1.4-2 of Volume 3 (AS) B.9.

In the present document, the study summaries of literature data considered by RMS as (less) relevant and reliable (with restriction) are presented. The document therefore contains also published literature studies considered that were considerd not relevant by applicant.

The RMS followed the following rules regarding the issues related to relevance and reliability.

No issues: The study is considered relevant and reliable. (endpoint should be considered in the risk assessment)

Relevance issues:

- Formulation-related issues
  - When there is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation (i.e. where the composition of the tested formulation is unclear). The study is considered less relevant but supplementary.
  - The tested formulation includes substances (POEA/…) that are not allowed in the EU (or similar to those substances) in accordance with (Regulation (EU) 2016/1313 and/or the DRAFT Regulation amending Annex III of Regulation (EC) 1107/2009), and therefore the results are not considered relevant for the assessment of the representative product. The study is considered not relevant
  - Since the tested formulation showed significantly higher toxicity compared to the parallel results from the active substance, RMS consider it likely that the tested formulation contains toxic co-formulants that are not present in the representative product. Therefore, the results from the formulation are not considered relevant for this assessment. (where active ingredient and a formulation are tested in the same study and the formulation is more toxic). The study is considered not relevant with regards to formulation results. Results with active substance are considered relevant.
- Other issues

For example unrealistic exposure route : The study is considered not relevant.

Reliability issues:

- For non relevant studies : Reliability assessment is not necessary. These studies will not be summarized. Indeed, according to Appendix of the literature search guidance, the "*References identified in literature search that are not considered relevant, should not be listed in the Assessment Report but only reported in the Table 1 with the reason of rejection*."
- Results of studies with lack of analytical verification were considered as reliable with restrictions.
- Results of studies with obvious drawbacks are considered not reliable.
- When reliability of the study cannot be evaluated based on the available information. The reliability will be reported as not assignable.

Regarding consideration for risk assessment purpose, studies fully relevant and reliable could be considered as fit for purpose for standard risk assessment. Studies less relevant (but supplementary) and/or reliable with restrictions was considered in weight of evidence.

B.9.1. EFFECTS ON BIRDS AND OTHER TERRESTRIAL VERTEBRATES

B.9.1.4. Other data on effects on terrestrial vertebrate wildlife (birds, mammals, reptiles and amphibians)

B.9.1.4.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

This section includes studies that were concluded by the RMS to be relevant, i.e., provide data for establishing or refining risk assessment parameters. These studies were summarised in detail by the applicant following the subsequent steps of the OECD Guidance documents (OECD, 2005; 2006). The studies have been further evaluated by the RMS and assessed for their reliability.

Data point	CA 8.2.8/001
Report author	Daam, M.A. *et al.*
Report year	2019
Report title	Lethal toxicity of the herbicides acetochlor, ametryn, glyphosate and metribuzin to tropical frog larvae
Document No.	doi.org/10.1007/s10646-019-02067-5 ISSN: 0963-9292
Guidelines followed in study	OECD (2015) Test No. 241: the larval amphibian growth and development assay ASTM (2013) Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX). ASTM E1439-12
Deviations from current test guideline	Not reported
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability as proposed by the applicant; See also RMS analysis in RMS box	Yes / Reliable with restrictions

The aim of this study was to evaluate the acute toxicity of the active ingredient glyphosate to tadpoles of two tropical frog species: *Physalaemus cuvieri* and *Hypsiboas pardalis*. The calculated 96 h LC_{50} (median lethal concentration; in mg a.s./L) values for *P. cuvieri* and *H. pardalis* were 115 and 106 mg a.s./L, respectively.

Materials and methods

Test species
Three or more egg masses from different parents of *Physalaemus cuvieri* and *Hypsiboas pardalis* were collected from ponds at the Estação Biológica de Boracéia in Salesópolis, South-East Brazil (23°37′59”S, 45°31′59”W), which is located within a non-polluted, protected watershed. Egg masses were transported in sealed plastic bags containing water from the collection site to the laboratory of the School of Arts, Sciences and Humanities in the University of São Paulo. Hatched larvae were kept in 50 L plastic tanks filled with tap water filtered through an activated carbon granular filter. Tank water was renewed every other day. The temperature in the laboratory was controlled at 25 ± 2 °C with natural photoperiod. Larvae were fed daily with a 3:1 ground mixture of rabbit chow (Purina Mills, LLC, USA; ~16% protein) and Tetra Min Fish Flakes (Tetra Werke, Melle, Germany; ~45% protein) ad libitum until the beginning of the experiments. The bioassays were conducted with Gosner stage 25 tadpoles.

Only healthy individuals, as judged by external morphology and behavior, were selected for the experiments.

Lethality tests
Acute (96 h) bioassays were conducted to evaluate the sensitivity of *P. cuvieri* and *H. pardalis* to the pure active ingredients glyphosate (CAS Number 1071-83-6; Purity 99.2%; Sigma-Aldrich). A semi-static design was adopted, in which test solutions were renewed 48 h after the start of the experiment.

The tests were conducted under the same conditions as those described above, except that animals were not fed during the test. Based on the results of range-finding tests, five logarithmically-spaced test concentrations (all in mg a.i./L) were determined: Glyphosate: 84; 97; 112; 130; 150.

Test concentrations were prepared with stock solutions. Each treatment was conducted in quadruplicate, in which each replicate consisted of a glass jar containing 10 tadpoles in 1 L test solution. Every 24 h, water quality parameters (pH, temperature, conductivity, DO) were recorded using a multi-parameter meter (YSI 556), and dead individuals counted and removed.

Data analysis
The 96h LC_{50}, LOEC (lowest observed effect concentration) and NOEC (no observed effect concentration) were calculated based on the % mortality rates in the different treatments using the statistical programs PROBIT 1.5 and TSK 1.5. In all cases, the most appropriate statistical test was defined depending on the experimental design and the nature of the available data, following the recommendations of EPA. To test for interspecies differences in sensitivity, LC_{50} values for each compound and species were compared with a Z test using the formula proposed by EPA. Analyses of Variance (ANOVA) followed by post hoc tests were employed to test for treatment effects on physical-chemical variables (mean values for each treatment over the experimental period) using the software PAST.

Results

Survival was 100% in all control treatments. Water quality parameters were comparable in control replicates with a coefficient of variation of less than 4% for all parameters (pH, temperature, conductivity and DO).
The 96h LC_{50} values generated and is presented in the table below, whereas the mortality levels of the individual treatments for *P. cuvieri* and *H. pardalis* are visualized in the Figures below.

Table 1: (from Daam, M.A. *et al.* 2019, Ecotoxicology Vol. 28, pp. 707–715): LC_{50} (median lethal concentration; in mg/L) and the 95% confidence interval as determined for larval *Physalaemus cuvieri* and *Hypsiboas pardalis* after 96-h exposure to glyphosate.

	Physalaemus cuvieri	Figure	*Hypsiboas pardalis*	Figure
Glyphosate	115 (112–119)[b]	1	106 (103–109)[b]	2

[b]Probit test

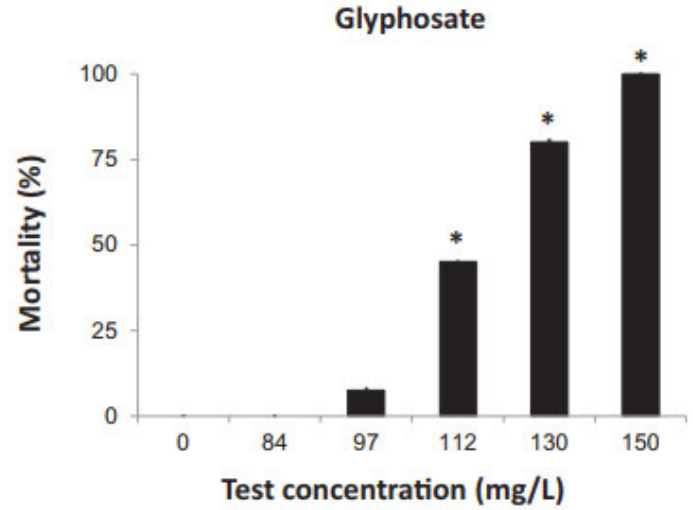


Figure 1: (from Daam, M.A. *et al.* 2019, Ecotoxicology Vol. 28, pp. 707–715): Mortality (in %) of *Physalaemus cuvieri* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

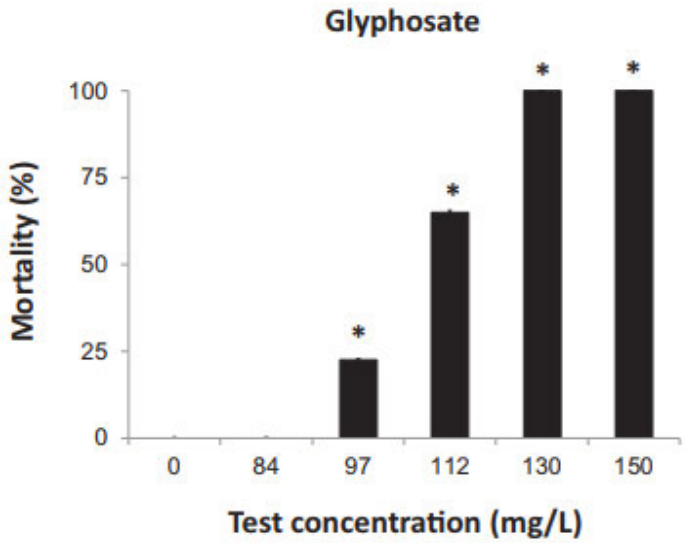


Figure 2: (from Daam, M.A. *et al.* 2019, Ecotoxicology Vol. 28, pp. 707–715): Mortality (in %) of *Hypsiboas pardalis* at the end of the 96 h laboratory tests evaluating the toxicity of glyphosate. Bars represent mean ± 1 SE of four replicates. Asterisks represent significant differences ($p < 0.05$) relative to the control.

The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

Assessment and conclusion

Assessment and conclusion by applicant:

The study investigated the acute toxicity of glyphosate to larvae of *Physalaemus cuvieri* and *Hypsiboas pardalis*. The LC_{50} for *Physalaemus cuvieri* and *Hypsiboas pardali* was determined to be 115 mg a.s./L and 106 mg a.s./L, respectively.

The study was conducted according to portions of OECD 241. However, validity criteria were not reported. It is unknown if the larvae were exposed to any other chemicals as no analysis of watershed water was provided. There was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

Assessment and conclusion by RMS:

The RMS agrees with the applicant's conclusion. The results are relevant for the risk assessment and the study is considered as reliable with restrictions.

Data point	CA 8.1.4
Report author	Turhan D. Ö *et al.*
Report year	2020
Report title	Developmental and lethal effects of glyphosate and a glyphosate-based product on *Xenopus laevis* embryos and tadpoles
Document No.	Bulletin of Environmental Contamination and Toxicology, (2020) Vol. 104, No. 2, pp. 173-179
Guidelines followed in study	ASTM (2003) American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-*Xenopus* (FETAX), E1439-98
Deviations from current test guideline	Jelly coats should be removed from developing embryos mid blastula stage embryos – ca. stage 8, prior to 96 hour exposure well plates. The removal is achieved using 2% cysteine solution followed by rinsing. No reference is provided in the article to see whether this step was completed (it is a relevant as it informs on ion-exchange and impacts from concentrated solution / osmotic and diffuse pressures on the embryos in the increasing concentrations of test media.) Test guideline followed for the late development stage 46 (96 hr test) exposure test was not stated. The FETAX assay runs until 80-90% of the control tadpoles reach developmental stage 47. This cannot be confirmed. Water quality at test start appears to be within specification, although after 96 hrs water quality data appeared to have been determined.
GLP/Officially recognised testing facilities	No
Acceptability/Reliability as proposed by the applicant; See also RMS analysis in RMS box	Yes / Reliable with restrictions

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late-stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

Information in the publication relating to a potassium salt-based glyphosate formulation was excluded from this summary as it cannot be related to the representative formulation MON 52276.

Materials and methods

Glyphosate was purchased from Sigma-Aldrich (PESTANAL®, analytic grade, 45521).

The embryos and tadpoles used in the tests were obtained from male and female frog pairs from an adult *X. laevis* colony in the Author's laboratory. *X. laevis* breeding and acquisition of embryos were performed according to ASTM-E1439-98 (ASTM 2003). Embryos and tadpoles were maintained in a standard Frog Embryo Teratogenesis Assay Xenopus (FETAX) test medium (ASTM 2003) with the following composition: 625 mg NaCl, 96 mg $NaHCO_3$, 75 mg $MgSO_4$, 60 mg $CaSO_4 \times 2H_2O$, 30 mg KCl, and 15 mg $CaCl_2$ per liter of distilled water.

The exposure solutions of glyphosate were prepared fresh daily in the standard FETAX test medium. The pH of glyphosate solutions was adjusted to 7.9, as recommended for FETAX tests, using NaOH. Embryos and tadpoles were exposed to test solutions under semi-static test conditions with a 12:12 h light:dark photo-period at 23 °C (± 1 °C).

Before starting the FETAX test, glyphosate levels in the test medium were measured using high-performance liquid chromatography (HPLC) (1100 system, Agilent Technologies, Santa Clara, CA, USA). The measured glyphosate concentrations in exposure media were determined to be at least 92% of the nominal concentrations.
For the FETAX test, stage 8 embryos were exposed to different glyphosate concentrations for 96 h. Four embryos were randomly selected, and placed with 2 mL of test medium into each well of 24-well plates, serving as one replicate per treatment. All concentrations were tested with eight replicates and thus a total of 32 embryos. In the FETAX test, seven concentrations of glyphosate (282–500 mg/L) were tested plus controls. The test medium was changed every 24 h. Dead tadpoles were removed and incidences were recorded. The median lethal concentrations (LC_{50}) were determined after 24, 48, 72, and 96 h of exposure. At termination of the bioassays, surviving embryos were euthanized and observed for developmental abnormalities.

For the tadpole-toxicity bioassays, stage 46 tadpoles were exposed to different glyphosate concentrations for 96 h. Five randomly selected tadpoles were placed in each well of 12-well plates containing 3 mL of test solution. All concentrations were tested with 6 replicates resulting in a total of 30 tadpoles per concentration. In the tadpole-toxicity tests, seven concentrations of glyphosate (250–403 mg/L) were tested excluding control groups.
For biochemical assays, stage 46 tadpoles were exposed to three glyphosate concentrations (50-250 mg/L) for an exposure period of 96 h. Fifteen randomly selected tadpoles and 10 mL of test solution were placed into 25 mL polycarbonate containers. All concentrations were tested with five replicates resulting in a total of 75 tadpoles per treatment. At the end of the exposure period, surviving tadpoles were euthanized and their enzyme activities were measured (glutathione S-transferase (GST), glutathione reductase (GR), carboxylesterase (CaE), acetylcholinesterase (AChE), superoxide dismutase (SOD)).

Graphpad Prism software (Version 5, USA) was used to calculate the average lethal concentration (LC50) and 95% confidence intervals (CI) and for other statistical analyses. A log(dose)-normalized response curve ($Y = 100/ (1 + 10(\log EC50 - x) \times \text{hillslope}$) to fit mortality data. For statistical analysis of biomarkers, data were tested initially for homogeneity of variances and normality distributions by the Bartlett and Kolmogorov-Smirnov tests, respectively. Nonparametric data were analyzed using Kruskal-Wallis test followed by pairwise comparisons of groups using Mann-Whitney U tests. Parametric data were analyzed using the One-way Analysis of Variance (ANOVA) followed by the unpaired t test. A Bonferroni correction was applied (0.05/3 = 0.016). In order to determine growth inhibition, the head-to-tail lengths were measured and the lengths were compared using ANOVA (Dunnett's post hoc test, $p < 0.05$).

Results

Even the highest glyphosate concentrations did not cause a lethality higher than 17% for both *X. laevis* embryos and tadpoles in this study. Moreover, the highest glyphosate concentrations (403 and 500 mg/L) caused no growth inhibition in embryos or tadpoles (see table below).

Table 1 (from Turhan D. Ö *et al.* 2020, Bull Environ Contam Toxicol, Vol. 104, No. 2, pp. 173-179): Length of stage 8 embryos and stage 46 tadpoles of *Xenopus laevis* after 96-h exposure to different concentrations of glyphosate.

Glyphosate					
Conc. (mg/L)	***n***	**Length (mm)**[a)]	**Conc. (mg/L)**	***n***	**Length (mm)**[a)]
8th stage			**46th stage**		
Control	32	7.14 ± 0.07	Control[b)]	30	10.13 ± 0.10
282	31	7.15 ± 0.08	250	25	10.52 ± 0.07
310	31	7.14 ± 0.07	275	29	10.36 ± 0.08
342	26	7.04 ± 0.08	303	26	10.44 ± 0.09
376	32	7.22 ± 0.06	333	29	10.28 ± 0.04
413	32	7.21 ± 0.08	367	26	10.23 ± 0.08
455	30	7.38 ± 0.08	403	25	10.48 ± 0.07
500	29	7.04 ± 0.10	---	---	---

[a)] Lengths are expressed as mean± standard errors. These values were obtained from the lengths of the surviving individuals (n).
[b)] The average length of tadpoles at the beginning of this test is 7.30 ±0.10 mm (n = 32).

In addition, the selected biochemical markers in tadpoles exposed to glyphosate did not show any statistically significant changes (see table below).

Glyphosate						
Concentration (mg/L)	***n***	**GST**[a)]	**GR**[a)]	**CaE**[a)]	**AChE**[a)]	**SOD**[b)]
Control	5	130 ± 3.9	11.2 ± 0.43	167 ± 4.4	142 ± 3.2	0.65 ± 0.05
50	5	134 ± 4.9	11.3 ± 0.38	155 ± 3.9	128 ± 6.9	0.60 ± 0.02
100	5	131 ± 1.0	9.7 ± 0.47	154 ± 2.5	126 ± 5.4	0.60 ± 0.05
250	5	141 ± 3.5	10.7 ± 0.28	170 ± 3.1	132 ± 6.5	0.64 ± 0.03

[a)] The enzyme activity was expressed as nmol/min × mg protein ± standard error.
[b)] The enzyme activity was expressed as U/mg protein ± standard error.

Effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as model system. No lethal or developmental effects were observed at all concentrations tested (up to 500 mg glyphosate/L). Measured biological parameters included growth (length) and measuring enzyme levels. Growth of tadpoles was measured after exposure of developing embryos in cell well plates for 96 hrs, exposed from developmental stage 8 for 96 hours. In a late stage tadpole exposure assay (from Stage 46) tadpoles were exposed to a range of concentrations prior to homogenising whole tadpoles for enzyme level analysis.

Assessment and conclusion

Assessment and conclusion by applicant:

Effects of pure glyphosate and a glyphosate-based product Roundup® Star (containing glyphosate in a form of a potassium salt and including 6% surfactant as ethoxylated alkylamine based, were evaluated comparatively using two embryonic development stages of the amphibian *Xenopus laevis* as model system. As the glyphosate-based product Roundup® Star is not the representative formulation for the European renewal of glyphosate, the summary only provides information for pure glyphosate.

However, this publication confirms a general trend that toxic effects caused by glyphosate-based products, compared to pure glyphosate, are increased mainly due to additives present in glyphosate formulations and that it may be a result of synergistic effects between glyphosate and adjuvant in the formulations.

In this study, no lethality >17 % or developmental effects (growth inhibition) were observed in embryos or tadpoles with pure glyphosate at any glyphosate concentration tested (282-500 mg/L for stage 8 embryos) and (250-403 mg/L stage 46 tadpoles).

In addition, no effect was observed with regards to enzymatic activity of stage 46 tadpoles at any glyphosate concentration tested (50-250 mg/L).

The article is classified as reliable with restrictions for the following reason: The specific purity of the test item was not reported. No OECD guidance has been followed. The American Society for Testing and Materials, Standard guide for conducting the Frog Embryo Teragonesis Assay-Xenopus (FETAX test), E1439-98 has been followed with some deviations to the recognised approach (see deviations above). The FETAX assay is a developmental toxicity screening test, which for the most part has been superseded by amphibian metamorphosis and developmental toxicity assays using *Xenopus laevis* (OECD 231 and OECD 241). Studies performed according to both of these recognised test guidelines were submitted with the Annex I dossier (M-CA Section 8.2.3/002 and M-CA Section 8.2.3/003). Whilst the FETAX assay is not directly recognised at the EU level, elements of the FETAX assay are considered in the conduct of the OECD 231 test guideline.

Control mortalities were not reported (only LC_{50} final results). Analytical verifications of the concentrations in the test medium were reported only before starting the test, but exposure medium was changed every 24 h to maintain the desired concentrations.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning for non-relevance of the study. Note that there is no requirement for studies from the open literature to follow OECD or any other standard protocol.

Since the tested formulation showed significantly higher toxicity compared to the parallel results from the active substance, we consider it likely that the tested formulation contains toxic co-formulants that are not present in the representative product. Therefore, the results from the RoundUp Star formulation are not considered relevant for this assessment.

96 h LC_{50} values for glyphosate could not be determined due to low mortality among both embryos and tadpoles (i.e., max 17% mortality). Thus LC_{50}>403 mg/L for tadpoles. NOEC for tadpole length is 403 mg/L and NOEC for enzymatic activity in tadpoles is 250 mg/L.

The study is reliable with restrictions, since analytical verification was only performed on test solutions from the FETAX test, not the tadpole toxicity bioassays.

Data point	CA 8.1.4
Report author	Bach N. C. *et al.*
Report year	2016
Report title	Effect on the growth and development and induction of abnormalities by a glyphosate commercial formulation and its active ingredient during two developmental stages of the South-American Creole frog, *Leptodactylus latrans*.
Document source	Environmental science and pollution research (2016), Vol. 23, No. 23, pp. 23959
Guidelines followed in study	US EPA 660/3-75-009

	ASTM E729-96 (2007)
Deviations from current test guideline	Minor modifications.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The acute lethal and sub-lethal effects of technical-grade glyphosate (GLY) and the GLY-based commercial formulation Roundup ULTRA MAX® (RU) on two Gosner stages (Gss) 25 and 36 of the South-American Creole frog, *Leptodactylus latrans*, were evaluated. Bioassays were performed following standardized methods within a wide range of concentrations (0.0007-9.62 mg of acid equivalents per liter (a.e./L) of RU and 3-300 mg/L of GLY). The endpoints evaluated were mortality, swimming activity, growth, development, and the presence of morphologic abnormalities, especially in the mouthparts. No lethal effects were observed on larvae exposed to GLY during either Gs-25 or Gs-36. The concentrations inducing 50% lethality in RU-exposed larvae at different exposure times and Gss ranged from 3.26 to 9.61 mg a.e./L. Swimming activity was affected by only RU. Effects on growth and development and the induction of morphologic abnormalities (like oral abnormalities and edema) were observed after exposure to either GLY or RU. Gs-25 was the most sensitive stage to both forms of the herbicide. The commercial formulation was much more toxic than the active ingredient on all the endpoints assessed.

Materials and methods

Chemicals
Test solutions were prepared with the GLY-based formulation Roundup ULTRA MAX® (Monsanto Argentina S.A.I.C., Maipú, Buenos Aires, Argentina), containing 74.7 % of the monoammonium salt of glyphosate (GLY acid equivalent to 67.9% w/w) and inert adjuvants *quantum satis*, and technical-grade GLY acid of 95.1% purity (Gleba, La Plata, Buenos Aires, Argentina). Dilutions were made from a 740-mg a.e./L stock solution for RU and a 1500-mg/L stock solution for GLY with filtered and dechlorinated tap water. The stock solution of GLY acid was adjusted to neutral pH. Samples of test solutions were taken at low, intermediate, or high concentrations, according to the experimental design, immediately after preparation (0 h) and after 24 h of exposure to confirm the concentrations. The GLY concentrations in test solutions were determined by LC/MS.

Test species
Small portions (about 10%) of five recently laid (8-10 h) foam nests of *L. latrans* were collected from temporary ponds from two well-preserved areas. Once in the laboratory, the organisms were maintained in tanks with 500 L of dechlorinated tap water with continuous aeration at 25 ± 1 °C and a 16:8 light/dark cycle. The larvae were fed *ad libitum* with blended lettuce until the individuals reached the stage needed. The tadpoles were maintained under laboratory conditions according to the Guide for Care and Use of Laboratory Animals (National Research Council 2011).

Experimental design: toxicity bioassays
The bioassays of toxicity were performed on tadpoles at two developmental stages: Gs-25 (±0) and 36 (±2) following standardized methods proposed by the US Environmental Protection Agency (1975) and the American Society for Testing and Materials (2007) with minor modifications by Natale *et al.* (2006).

Toxicity bioassays with Gosner-stage 25
The bioassays were carried out in glass chambers with five individuals and 500 mL of the corresponding test solution under semi static conditions (with medium replacement every 24 h) at four replicates per

concentration. Since a 72-h starvation could become a significant condition in the survival of young tadpoles, two types of toxicity bioassays were performed - involving feeding and non-feeding - to compare the effects under both experimental conditions. Tadpoles were fed with 1 mL of blended lettuce every 24 h, 1 h before medium replacement, in the feeding bioassay and were not fed throughout the experiment in the non-feeding bioassay. Preliminary tests were performed in order to arrive at a wide GLY-concentration range for assessing lethal and sub-lethal effects. Definitive bioassays were conducted with 23 concentrations, ranging from 0.0007 to 9.62 mg a.e./L of RU (encompassing both sub-lethal and lethal concentrations), 7 concentrations between 3 and 300 mg/L of GLY, and a control group with merely dechlorinated tap water.

Toxicity bioassays with Gosner-stage-36 tadpoles
Bioassays were performed based on the results of Gs-25 tests, with seven concentrations between 0.37 and 9.62 mg a.e./L of RU (involving both lethal and sub-lethal concentrations) and seven concentrations between 3 and 300 mg/L of GLY (those being only sub-lethal concentrations). Once 50% of the tadpoles reached Gs-36, the individuals were placed in test chambers according to the experimental procedure cited above. Testing conditions were the same as those explained for bio-assays with the Gs-25 larvae. The tadpoles were not fed throughout the bioassays with the Gs-36 larvae because those individuals did not manifest indications of starvation during the later stages.

Endpoints measured
Mortality was evaluated every 24 h and determined by the absence of movement after gently prodding the tadpoles with a polypropylene rod as well as by the change in their color and overall appearance. Dead individuals were removed and fixed in 10% (v/v) aqueous formaldehyde.

Swimming activity was registered every 24 h by gently swirling the water three times with a polypropylene rod and observing for 1 min the swimming of each individual. The effects on swimming were classified according to three categories: regular swimming, irregular swimming (erratic swimming, body twisting, and convulsions), and immobility (complete stillness for the whole observation period, but with slight movement observed after gently prodding with the propylene rod).

At the end of the experiments, all the tadpoles still alive were anesthetized and finally preserved in 70% (v/v) aqueous ethanol for subsequent evaluation of growth, development, and the occurrence of morphologic deformities. Growth was determined by measuring the body length, i.e., snout-vent length (SVL). Development and morphologic characteristics were observed, the stages of development identified, and the abnormalities classified. 14 types of deformities were considered.

Statistical analysis
A regression analysis was performed between nominal and measured concentrations of GLY in the water, and the regression coefficient (b) was accordingly used to correct the concentrations presented in this study. The measured concentrations at the initial time (0 h) and after 24 h were compared by a paired Student *t*-test. Each swimming category was measured and recorded as a binary response (i.e., present or absent). Mortality and swimming activity data were analyzed by the Probit method (Finney 1971), in order to estimate LC_{50} and the concentrations producing 50% effect (EC_{50}), respectively. Concentration response curves at different times (24, 48, 72, 96 h) were estimated along with their 95% confidence limits. Regression (a and b) and correlation (r) coefficients were calculated for each curve and comparisons between different regression lines made. In addition, the swimming activity, growth, and abnormality data were analyzed by a one-way ANOVA with the Dunnett post-hoc test to estimate the concentrations for no observed effect (NOEC) and the lowest observed effect (LOEC). Effects on development compared to control group were evaluated by means of the Kruskal-Wallis test along with the Dunn post-hoc test. The incipient lethal concentration was estimated (according to LC_{50} values) as the point at which the curve began to run parallel to the x-axis. Comparisons between the LC_{50} values for the conditions of feeding and non-feeding were made with a paired Student t test and between the lethal C-R curves for the Gs-25 and Gs-36 at each exposure time by simple linear regression comparison. Comparisons of the sub lethal data at 96 h between GLY and RU bioassays or between the Gs-25 and Gs-36 were made with a paired Student *t*-test.

Results

Chemical analysis
The GLY concentrations are given after corrections were made based on the measurements on the test solutions of RU and GLY. Moreover, the concentrations measured at the initial time (0 h) and after 24 h were not significantly different ($p = 0.507$), thus indicating that the GLY concentrations remained constant throughout the bioassays.

Lethal effects
Table 1 summarizes the lethal effects of RU on Gss 25 and 36 of *L. latrans* tadpoles. Because of the high mortality at 96 h in the non-fed group, the results demonstrate that *L. latrans* requires feeding during the early stages of development, though such feeding is not necessary if tests are performed with more advanced developmental stages.

In contrast, the paired t-test of the LC_{50} values between the fed and non-fed groups after 24, 48, and 72 h of exposure indicated no significant differences, thus suggesting that food supply does not influence the lethal effects of GLY under conditions of acute exposure. The results support the absence of toxicity by GLY at up to 300 mg/L for both the Gs-25 and Gs-36 larvae, since the authors observed no lethal effects upon treatments with GLY (Table 2).

The formulation RU exhibited toxic effects with LC_{50} values at different exposure times and Gosner stages that ranged from 3.26 to 9.61 mg a.e./L (cf. Table 1 and Table 2).

In the present work, a multiple comparison of the various C-R curves for Gs-25 larvae exposed to RU for different lengths of time indicated significant differences except between 72 and 96 h. The data suggest that commercial formulations of GLY exhibit a lethal effect within the first days of exposure and reach the incipient value after 72 h (Fig. 1). Comparisons among C-R curves between Gs-25 and Gs-36 tadpoles exposed to RU revealed significant differences at 72 and 96 h of exposure, indicating that Gs-25 was more sensitive to toxicity by RU than Gs-36 (Table 1).

Table 1 (from Bach N. C. *et al.* 2016, ESPR, Vol. 23, No. 23, pp. 23959). Lethal effects of RU on Gs-25 and Gs-36 *L. latrans* larvae.

Time (h)	LC_{10} (95 % CI)	LC_{50} (95 % CI)	LC_{90} (95 % CI)	a	b	*r*
"Feeding" Gosner-stage 25						
24	5.48 (5.00–5.81)	6.76 (6.45–7.11)	8.36 (7.84–9.22)	–11.63	17.30	0.94
48	2.90 (2.48–3.18)	3.86 (3.60–4.11)	5.15 (4.78–5.81)	–1.29	9.22	0.91
72	2.80 (2.36–3.03)	3.28 (3.03–3.46)	3.84 (3.63–4.25)	–1.90	11.63	0.94
96	2.84 (2.46–3.05)	3.26 (3.04–3.43)	3.74 (3.54–4.09)	–3.34	13.81	0.93
"No feeding" Gosner-stage 25						
24	5.97 (5.42–6.31)	7.13 (6.80–7.55)	8.50 (7.92–9.75)	–16.68	22.22	0.97
48	4.71 (4.25–5.02)	5.70 (5.42–5.97)	6.90 (6.51–7.58)	–7.41	14.25	0.93
72	3.09 (1.82–3.71)	4.17 (3.27–4.65)	5.62 (5.06–6.79)	–1.42	9.59	0.66
"No feeding" Gosner-stage 36						
72	7.84 (5.94–8.52)	9.61 (8.92–11.45)	11.78 (10.43–19.75)	–8.17	11.80	0.98
96	7.40 (6.35–7.94)	8.67 (8.12–9.28)	10.16 (9.45–11.91)	–10.90	14.90	0.98

Time indicates length of exposure. $LC_{10/50/90}$ and 95 % confidence limits in mg a.e./L
a intercept, *b* slope, *r* correlation coefficient

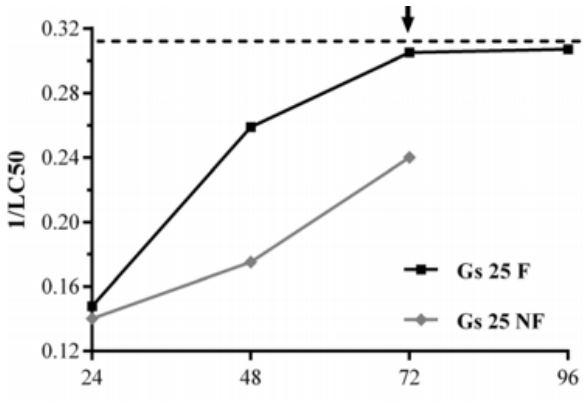


Figure 1 (from Bach N. C. *et al.* 2016, ESPR, Vol. 23, No. 23, pp. 23959). Incipiency curve ($1/LC_{50}$ vs. exposure time) for Gs-25 larvae exposed to RU in fed (F) and nonfed (NF) bioassays. The arrow indicates the incipient $1/LC_{50}$.

Sub-lethal effects

Swimming activity was affected by exposure to only RU, while effects on growth, development, and abnormalities were observed for both GLY and RU. A paired t-test between LOEC values for sub-lethal effects of both GLY and RU bioassays showed significant differences, indicating that RU was definitively more toxic than GLY. In addition, a paired t-test between sub-lethal NOEC values for tadpoles of those same stages showed significant differences, indicating Gs-25 tadpoles to be more sensitive than those of Gs-36 in all the endpoints tested. Because of the low number of survivors, and the failure of larvae to tolerate 96 h of exposure in the non-fed-group bioassays, sub lethal effects were examined only for bioassays of the fed group.

Effects on swimming activity

No effects on the swimming activity were observed in either Gs-25 or Gs-36 tadpoles exposed to GLY (Table 2).

In the present study, a one-way ANOVA performed on the swimming activity data indicated that irregular swimming was observed in Gs-25 larvae after 24 h of exposure with a LOEC value of 5.92 mg a.e./L, although no significant effects were observed upon exposure of Gs-25 tadpoles subsequent to that time. In addition, irregular swimming was also noted in Gs-36 larvae exposed to RU with a 96-h EC_{50} of 7.99 mg a.e./L (7.63-31.07). Swimming activity data on the Gs-36 tadpoles revealed significant differences with respect to irregular swimming, with a LOEC value of 5.18 mg a.e./L.

In summary, the results have demonstrated that tadpoles of different Gosner stages were affected in their ability to swim.

Table 2 (from Bach N. C. *et al.* 2016, ESPR, Vol. 23, No. 23, pp. 23959). Summary of endpoints evaluated on two Gosner-stages of *L. latrans* exposed to GLY and RU.

	GLY				RU			
	Gs-25		Gs-36		Gs-25		Gs-36	
	Cg	Treatment	Cg	Treatment	Cg	Treatment	Cg	Treatment
Lethal effects								
Mortality	ND	ND	ND	ND	0.05	1.0a	ND	0.80a
Sublethal effects								
Swimming Activity	ND	0.10 ± 0.12	0.10 ± 0.19	0.20 ± 0.20	ND	0.31 ± 0.25[a]b	0.08 ± 0.16	0.33 ± 0.11[a]
Growth (SVL)	5.69 ± 0.63	6.54 ± 0.86[a]	13.15 ± 2.33	11.66 ± 3.24	4.21 ± 0.34	4.69 ± 0.33[a]	13.56 ± 1.92	12.78 ± 0.30
Development (Gs)	27.53 ± 0.62	28.15 ± 0.67[a]	36.13 ± 2.64	35.63 ± 3.96	25.95 ± 0.23	26.56 ± 0.51[a]	36.40 ± 1.76	35.77 ± 1.64
Abnormalities								
Edemas	0.06 ± 0.12	0.10 ± 0.20	ND	0.69 ± 0.30[a]	ND	0.68 ± 0.36[a]	0.06 ± 0.11	0.27 ± 0.23
Oral disk	ND	0.52 ± 0.13[a]	0.13 ± 0.11	0.53 ± 0.23[a]	ND	ND	0.20 ± 0.20	0.67 ± 0.11[a]

Mortality data are expressed as the fractional proportion of dead individuals. The swimming activity and abnormality data are expressed as the mean proportion of the effect ± SD. Growth data are expressed as the mean snout-vent length (SVL; in mm) ± SD. Developmental data are presented as the mean Gosner stages ± SD. The lowercase letter *a* indicates the proportion of dead tadpoles at the highest concentration tested. All data correspond to 96 h of exposure except for the lowercase letter *b* indicating effects at 24 h of exposure

ND no effects detected, *Cg* control group

[a] Correspond to mean value ± SD for the LOEC concentration

Effects on growth and development

In this study, a one-way ANOVA demonstrated that larvae at Gs-25 exhibit a significant increment in growth after exposure to GLY with a LOEC value of 15 mg/L. In contrast, no significant effect on growth was observed for Gs-36 tadpoles when exposed to GLY (Tables 2 and 3).

In comparison, a test of the effect of exposure to RU on the growth of Gs-25 larvae by a one-way ANOVA indicated a significant difference, with a LOEC value of 0.37 mg a.e./L. The same one-way ANOVA performed on the growth data for the Gs-36 larvae showed no significant effects by RU (Tables 2 and 3).

The results on the development of Gs-36 individuals showed no significant effects after exposure to GLY, though differences were observed in Gs-25 larvae exposed to the compound, resulting in a significant increase in growth over that of the control group, with a LOEC value of 15 mg/L (Table 3).

In the present study, although Gs-36 larvae exposed to RU evidenced no significant differences in development, Gs-25 tadpoles similarly exposed manifested a significant increment in the rate of metamorphosis with a LOEC value of 0.0007 mg a.e./L (Table 3). The authors observed that exposure to either GLY or RU at early developmental stages induced an impairment of growth and further development. In this regard, the results could be indicating that GLY can be considered a stress-producing agent for tadpoles by causing an accelerated development that reaches metamorphosis at a precocious age. Within this context, the absence of an effect on Gs-36 individuals as opposed to the developmental acceleration of Gs-25 larvae can be explained by the minimum body size required to achieve metamorphosis.

Table 3 (from Bach N. C. *et al.* 2016, ESPR, Vol. 23, No. 23, pp. 23959). Sub-lethal effects (LOEC values in, mg/L of GLYand mg a.e/L RU) on two Gosner stages of *L. latrans* larvae after 96 h of exposure.

Effect	Gs-25		Gs-36	
	RU	GLY	RU	GLY
Development	0.0007	15	ND	ND
Growth	0.37	15	ND	ND
Abnormalities	2.96	30	2.22	30
Swimming activity	ND	ND	5.18	ND

ND no effects detected

Morphologic abnormalities
The proportion of abnormalities observed in the control group in all the bioassays was below 10%, except for the loss of tooth rows in Gs-36 larvae observed in 20 % of the controls. The results indicated that GLY induced a loss of upper- and/or lower-jaw sheaths in tadpoles of Gs-25 and Gs-36, with the same LOEC value of 30 mg/L being detected for both Gosner stages. Moreover, a one-way ANOVA with Gs-36 tadpoles revealed significant differences in the incidence of edema from 300 mg/L of GLY.

A one-way ANOVA revealed that the presence of edema in Gs-25 tadpoles exposed to RU exhibited significant differences in occurrence from control values, at a LOEC value of 2.96 mg a.e./L. In addition, Gs-36 data demonstrated significant differences in the incidence of tooth-ridge abnormalities and the loss of tooth rows from control values, with LOEC values of 2.22 mg a.e./L for both determinations. Please refer to Tables 2 and 3.

Conclusion

Experimental use of the species *L. latrans* enabled the detection of adverse effects induced by exposure to a formulation of GLY and to the active form of the herbicide. Lethal and sub-lethal effects were reproducible under standardized laboratory conditions.

The GLY-based formulation Roundup ULTRA MAX® induced acute lethal and sub-lethal effects on *L. latrans* tadpoles. According to the categories established by the US Environmental Protection Agency, the Roundup ULTRA MAX® formulation can be classified as a slightly toxic (class III) agent, while GLY can be classified as a practically non-toxic (class IV) compound.

Both forms of the herbicide induce similar sub-lethal effects, though at different concentrations, with RU being five orders of magnitude more toxic than GLY. This difference suggests the conclusion that adjuvants in the formulation may favor bioavailability of the active ingredient or else also consist of additives that contribute to toxicity.

The worst-case scenario that has been detected of 0.7 mg a.e./L, a level at which sub-lethal effects on growth and development and the induction of morphologic abnormalities were observed.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The data on the formulation are not relevant for the glyphosate EU renewal (formulation used is not the representative formulation MON 52276). There are no endpoints stated for the technical material in the paper that could be used in an ecotoxicological regulatory risk assessment / glyphosate EU renewal. Lethality endpoints are only inferred in Table 2. For lethality at both growth stages, 'ND' appears in the Table 2, suggesting a lethality endpoint that is beyond 300 mg technical material /L – the highest rate tested with the technical material. The test duration over which this observation was achieved is not stated / unknown. The two guidelines followed, recommend a test duration of 96 hours – however, there is no specific instruction in these test guidelines for this species of amphibian. Currently recommendation for amphibian larval testing is to expose for at least 96 hours. Presence / absence of food during the technical test is unknown. Only results for comparative tests (feed / no feed) with the formulation are presented (Table 1) not for the technical material. The robustness of the test system for eliciting development and inducing morphological deformities cannot be confirmed as there was no positive control group used in the test system. Uncertainty over the impact of the limitations of the test design on the anatomical observations cannot be excluded. No chemical analysis data are presented, therefore exposure cannot be confirmed. The statement in the results that concentrations at 0 and 24 h were not significantly different does not confirm exposure. There are no water quality data presented covering the exposure period, therefore the influence of water quality on the anatomical observations cannot be excluded. The study is considered unreliable.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning for regarding this study as supplementary. Some of the arguments listed are inaccurate, namely that exposure was not confirmed (chemical analysis was performed) and that exposure time for mortality observations is not stated (it is stated, 96 h). In addition, the lack of water quality data does not make the study less reliable, as any potential impact of water quality on the test organisms would also be seen in the controls. Thus, the RMS considers this study relevant and reliable.

Glyphosate technical up to the highest tested concentration of 300 mg/L did not cause any tadpole mortality during 96 h of exposure. LOEC for development and growth of Gs 25 larvae are both 15 mg/L, while LOEC for morphological abnormalities is 30 mg/L for both Gs 25 and Gs 36.

There is not sufficient information in the study report to demonstrate whether the tested formulation **Roundup ULTRA MAX®** is comparable with the representative product. However, Roundup ULTRA MAX® was tested in parallel with the active substance glyphosate and showed significantly higher toxicity than the latter, as well as significantly higher toxicity than observed in studies on the representative product. The RMS thus considers it likely that the tested formulation contains toxic co-formulants that are not present in the representative product MON 52276. Therefore, the results from Roundup ULTRA MAX® are not considered relevant for this assessment.

Data point	CA 9
Report author	Babalola O. O. *et al.*
Report year	2019
Report title	Mortality, teratogenicity and growth inhibition of three glyphosate formulations using Frog Embryo Teratogenesis Assay-Xenopus
Document No.	Journal of applied toxicology (2019), Vol. 39, No. 9, pp. 1257-1266
Guidelines followed in study	Protocol from Dawson & Bantle, 1987
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The study aimed to assess the embryotoxicity and teratogenicity of three glyphosate-based formulations using the 96-hour Frog Embryo Teratogenesis Assay-*Xenopus* protocol. Embryos of *Xenopus laevis* were exposed to Roundup, Kilo Max and Enviro Glyphosate at concentration of 0.3-1.3, 130-280 and 320-560 mg acid equivalent (a.e.)/L respectively. The results showed Roundup to be more toxic than the other formulations with a 96-hour LC_{50} of 1.05 mg a.e/L. compared with 207 mg a.e./L, and 466 mg

a.e./L for Kilo Max and Enviro Glyphosate respectively. Although, both Roundup and Kilo Max formulations show inhibition on growth of the embryo-larva ($P < .05$), the minimum concentration inhibiting growth ratios of the three formulations was >0.30 baseline, indicating no significant growth inhibiting effect in the formulations. For teratogenicity, Roundup and Enviro Glyphosate formulations exhibited increasing teratogenic traces, with the teratogenic index at 1.7 and 1.6 respectively. Kilo Max formulation shows low teratogenicity with the teratogenic index at 1.4. Characteristic malformation induced by these formulations included generalized edema, cardiac and abdominal edema, improper gut formation and axial malformations.

Materials and methods

Test material:	Three glyphosate-containing herbicides: - Roundup (360 g/acid equivalent (a.e.)/L containing POEA surfactant, Monsanto, S/Africa Ltd), - Enviro Glyphosate (360 g a.e/L, containing polyethylene alkylamine surfactant., Enviro Industries Ltd), - Kilo Max (700 g a.e/kg, equivalent to 791 g/L glyphosate sodium salt, containing undisclosed surfactant, Volcano Agro-science Ltd)
Exposure concentrations:	Roundup - 0, 0.5, 0.7, 0.9, 1.1, 1.3 mg a.e./L Enviro Glyphosate - 0, 320, 360, 400, 440, 480, 520, 560 mg a.e./L Kilo Max - 0, 130, 160,190, 220, 250, 280 mg a.e./L
Test organism:	
Species:	*Xenopus laevis*
Size:	Not stated
Age:	Developing embryos from mature male and females induced to breed in lab.
Source:	Commercial hatchery - Pinzas Rojas S.R.L, Tucuma´n, Argentina
Test design:	Semi-static (daily renewal) 96-hour Frog Embryo Teratogenesis Assay-Xenopus protocol, embryotoxicity and teratogenicity assay (Dawson & Bantle, 1987). Fertilised eggs Nieuwkoop and Faber (1994) stages 8-11 (mid blastula to early gastrula embryos) were collected and de-jellied by gentle swirling in 2% L-cysteine.
Test duration:	96 hours
Replication:	2 replicates per treatment group + 4 replicates for the control group.
Animal per replicate:	20 developing embryos per replicate
Test volume (L):	500 mL glass bottle.
Loading:	20 embryos per 500 mL
Feeding:	Not applicable
Chemical analysis:	Four random samples from each of the formulations were sampled and analyzed to confirm the experimental concentrations. The glyphosate analyses and quantification were performed using liquid chromatography-tandem mass spectrometry. The analysis was done by the Synexa Life Sciences certified laboratory. The results of the analysis showed that there was no major difference to the predicted nominal concentrations.
Environmental conditions:	Water temperature 24 ± 2°C, pH 6.5-7.4, dissolved oxygen of >6.5 mg/L and 12 hours light and dark photoperiod (L12: D12)

Observations

Mortality
Data in the exposure were monitored and taken every 6 hours. All the dead embryos were removed instantly. The exposure was terminated after 96 hours, and the final dataset used to determine the 96-hour lethal concentration (LC) at three different levels (LC_5, LC50 and LC95). The surviving tadpoles were killed in MS 222 (Tricaine methane sulfonate) (200 mg/L buffered with sodium bicarbonate at 0.42-1.05 g/L) (OECD, 2007). All the tadpoles used for the exposure were preserved in 4% formaldehyde for morphological assessment.

Growth inhibition
The lowest-observed-effect-concentration (LOEC) was derived by comparing the total lengths of the exposed embryos to that of the control individuals. In particular, the lowest concentration per formulation, which resulted in a significant reduction in total length, equates the LOEC. The minimum concentration inhibiting growth (MCIG) was derived as LOEC/LC50, showing the degree of growth inhibition independent of acute toxicity.

Malformation
Developmental malformations were defined according to the Atlas of Abnormalities by Bantle, Dumont, Finch and Linder (1999) and include facial as well as axial malformations (tail, and notochord abnormalities). Malformations were identified in both the deceased individuals and those that survived the 96-hour exposure. The incidences of abnormalities (%) were used to determine the 96-hour malformation (EC_{50}) index. The characteristic malformation assessment method was adopted following Fort and Paul (2002).

Teratogenic index (TI)
Was taken as the ratio of the 50% embryo lethality (LC_{50}) relative to the concentration causing 50% malformation (EC_{50}) (Dawson & Bantle, 1987). These two point-estimates were used to calculate the TI, i.e., TI = LC/EC (ASTM, 2014).

Statistical analysis
Mortality and malformation data were used to generate LC_5, LC_{50}, LC_{95} and EC_{50} estimates using the USEPA Probit analysis program (USEPA 1998). Normality of the data and homogeneity of variance was assessed using Shapiro-Wilk and Levene's test respectively. The variance within datasets was explored using one-way ANOVA or Kruskal-Wallis ANOVA. Pairwise differences were assessed using the Tukey-HSD post hoc or Dunn's multiple comparisons tests. $P < .05$ were deemed significant. Statistica v 13 (Dell Software Inc.) was used for statistical analyses.

Results

Embryo lethality/mortality
Roundup was the most embryo-lethal, of the three formulations tested, with a markedly lower 96-hour LC50, followed by Kilo Max and Environ Glyphosate (Table 1). (The calculated 96-hour LC_{50} for Roundup, Kilo Max and Environ Glyphosate were 1.05 ± 0.91- 1.32 mg a.e./L, 207 ± 197-218 mg a.e./L and 466 ± 434-512 mg a. e./L respectively.)

Table 1 (from Babalola O. O. *et al.* 2019, J. Appl. Toxicol., Vol. 39, No. 9, pp. 1257-1266). Concentrations, 96-hour LC_5, LC_{50} and LC_{95}, EC_{50}, LOEC, MCIG and TI, for Roundup, Kilo Max and Enviro Glyphosate (95% CI in bracket).

Treatment	Exposure concentration (mg a.e./L)	LC_5 (95% CI) (mg a.e./L)	LC_{50} (95% CI) (mg a.e./L)	LC_{95} (95% CI) (mg a.e./L)	EC_{50} (95% CI) (mg a.e./L)	LOEC (mg a.e./L)	MCIG (LOEC/LC_{50})	TI
Roundup	0, 0.5, 0.7, 0.9, 1.1, 1.3.	0.4 (0.24-0.53)	1.05 (0.91-1.32)	2.67 (1.88-5.99)	0.60 (0.36-0.81)	0.9	0.86	1.7
Kilo Max	0, 130, 160,190, 220, 250, 280	163 (143-175)	207 (197-218)	264 (247-296)	150.8 (139-160)	190	0.92	1.4
Enviro Glyphosate	0, 320, 360, 400, 440, 480, 520, 560	277 (196-322)	466 (434-512)	784 (655-1198)	287 (143-320)	440	0.94	1.6

LOEC, lowest observed effect concentrations; MCIG, minimum concentration inhibiting growth; TI, teratogenic index.

Growth effects and minimum concentration inhibiting growth

The total body length (TBL) of embryo-larva exposed to Roundup varied significantly among the treatment concentrations ($P < 0.05$), with a significant reduction in TBLs associated with higher dosage (0.9, 1.1 and 1.3 mg a.e./L) relative to the control (Fig. 1). The LOEC for Roundup was 0.9 mg a.e/L (Table 1), producing MCIG of 0.86 when normalized to the 96-hour LC_{50} of 1.05 mg a.e/L. For the Kilo Max formulation, there was a significant and concentration-dependent reduction in TBL ($P < 0.05$). The reductions in TBL were significant from the control at concentrations of 190-280 mg a.e./L ($P < 0.05$) (Fig. 1). The LOEC for the Kilo Max was 190 mg a.e./L (Table 1), which equate to an MCIG of 0.92 when normalized to the 96 hour LC_{50} of 207 mg a.e/L. Exposure of embryo-larva to the Enviro Glyphosate formulation resulted in a significant ($P < 0.05$) TBL reduction at 440 mg a.e./L compared with the control tadpoles (Fig. 1). The LOEC for this formulation was 440 mg a.e/L, which produced an MCIG of 0.94 (Table 1).

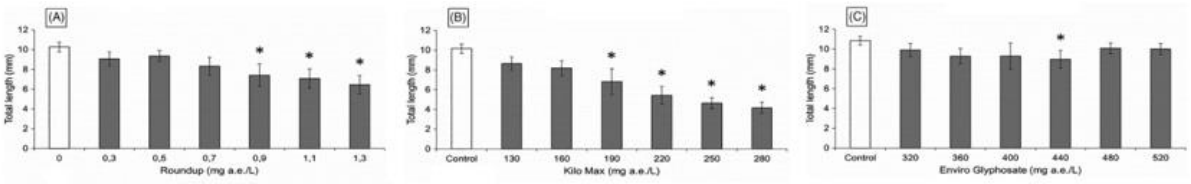


Figure 1 (from Babalola O. O. *et al.* 2019, J. Appl. Toxicol., Vol. 39, No. 9, pp. 1257-1266). Length (mean ± SD) of *X. laevis* after treatment with for 96 h. A, Roundup. B, Kilo Max. C, Enviro Glyphosate. Asterisks indicate significant differences relative to the control ($P < 0.05$).

Effective concentration (malformations) (EC50) and teratogenic index

As was the case with LC_{50}, the EC_{50} for malformations associated with Roundup exposure was lower than calculated for Kilo Max and Environ Glyphosate. In particular, the 96-hour EC50 for malformations obtained for Roundup was 0.6 mg a.e./L, and the EC 50 for Kilo Max and Enviro Glyphosate was 150.8 mg a.e./L and 287 mg a.e./L respectively (Table 1). The TIs obtained were 1.7, 1.4, and 1.6 for Roundup, Kilo Max and Enviro Glyphosate respectively (Table 1).

Specific observed malformations

A range of different malformations were recorded in all formulations. The observed characteristic malformations associated with exposure to the Roundup formulation include improper gut coiling, edema (severe generalized, abdominal and cardiac; blistering, as well as head (oblong and sloppy head shape occurring at the concentration around the EC_{50}. Some of the malformations occurred together in a single organism. The percentage incidences of various malformation categories for the Roundup formulation were recorded in the following order: gut abnormalities (42.5%); generalized edema (22.6%); blistering (22.2%); abdominal edema (5.4%); cardiac edema (3.6%); head (2.3%); and eye (1.4%). Exposure to the Kilo Max formulation series produced characteristic malformations, including edema (severe generalized, abdominal and cardiac; improper gut coiling, axial malformations, blistering and eye malformations (no pigment and choroid partial closure) occurring at concentrations around the EC_{50}. While some of the malformations occurred together in a single organism, the percentage incidences of various malformation categories were in the following order: gut abnormalities (32.5%); generalized edema (29%); axial abnormalities (22.5%); abdominal edema (8%); blistering (4%); eye (2.2%); head (1.3%); and cardiac edema (0.9%). The observed characteristic malformations in the tadpoles exposed to the concentration series of the Enviro Glyphosate formulation include incomplete gut coiling, edema (severe generalized, abdominal and cardiac; axial malformations (including wavy and curved tails; and reduced head and brain size, occurring at concentrations around the EC_{50}, while eye malformations (optic cup rupture and choroid rupture occurred at concentrations far above the LC_{50}. The percentage incidences of various malformations were recorded in the following order: gut abnormalities (34%); abdominal edema (31.6%); cardiac edema (20.9%); axial abnormalities (wavy and curved tail) (6.4%); head (2.1%); and eye (1.6%).

Conclusion

The present study shows that Roundup is moderately lethal to the *X. laevis* embryo-larval developmental stage, while Kilo Max and Enviro Glyphosate have relatively low embryo lethality. The results therefore suggest that the embryo lethality induced by the Roundup formulation may be linked to the surfactant in the formulation, as Enviro Glyphosate and Kilo Max formulations showed no significant lethality on the embryo-larval stages of *X. laevis*. In terms of teratogenicity, Roundup and Enviro Glyphosate showed increasing teratogenic potential, while Kilo Max and Roundup formulations only showed positive dysmophogenic effects. This suggests that the teratogenic potential associated with the Roundup and Enviro Glyphosate formulations may be attributed to their respective surfactants. Therefore, the contribution of surfactants to the teratogenicity, dysmorphogenicity and toxicity calls for more research effort to confirm their exposure impacts when used in formulations with glyphosates. Future formulation development should consider less toxic and environmentally friendly surfactants. This present study also confirms that pesticide contamination in the aquatic environment as one of the potential drivers of the global amphibian decline cannot be ruled out. The importance of embryo-larval lethality assessment during the early stage (first tier) must therefore not be underestimated, particularly when screening the formulations as applied in practice, and not just the active ingredient, as currently performed. This information is valuable when planning investigations, including additional functional endpoints, e.g., when testing the endocrine-disruption hypothesis.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The study presents endpoints that are not related to the EU level ecotoxicological regulatory risk assessment, as the techniques used are not recognised at the EU level.

In addition, none of the formulations tested is the representative formulation MON 52276 for the glyphosate EU renewal.

Further points of clarification:
Although Roundup formulation was tested, it does however contain different surfactant system than the representative formulation MON 52276. The surfactant system used in the formulation tested in this paper is polyethoxylene amine based (POEA), whereas the surfactant used in MON 52276 is quaternary-ammonium based. POEA surfactant is not permitted in formulated herbicidal products in the EU.

The Enviro glyphosate formulation contains polyethylene alkylamine surfactant, whereas the surfactant used in MON 52276 is quaternary-ammonium based.

Kilo Max contains 700 g a.e/kg, equivalent to 791 g/L glyphosate sodium salt, whereas the representative formulation for the glyphosate EU renewal MON 52276 has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L and is formulated using glyphosate isopropyl-ammonium salt.

Assessment and conclusion by RMS:

The results for the tested Roundup formulation are not considered relevant for the assessment of the representative product since the tested formulation includes surfactants that are similar to substances not allowed in the EU (Regulation (EU) 2016/1313 and/or Regulation (EU) 2021/383.

However, the Enviro formulation does not contain any substances forbidden within the EU and is thus relevant for the risk assessment, whereas for Kilo Max there is not sufficient information in the study report to demonstrate whether this formulation is comparable with the representative formulation. Therefore, the results on Kilo Max are considered less relevant but supplementary and will be used in a WoE approach.

The 96 h LC_{50} for *Xenopus laevis* embryos for Enviro and Kilo Max are 446 and 207 mg a.e./L, respectively.

The RMS considers this study to be reliable.

Data point	CA 9
Report author	Lajmanovich R. C. *et al.*
Report year	2011
Report title	Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (Anura: Bufonidae) tadpoles: B-esterases and glutathione S-transferase inhibitors
Document No.	Archives of environmental contamination and toxicology (2011), Vol. 60, No. 4, pp. 681-9
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

In this study, amphibian tadpoles *Rhinella arenarum* were exposed to different concentrations of Roundup Ultra-Max (ULT), Infosato (INF), Glifoglex, and C-K YUYOS FAV. Tadpoles were exposed to these commercial formulations with glyphosate (CF-GLY) at the following concentrations (acid equivalent [ae]): 0 (control), 1.85, 3.75, 7.5, 15, 30, 60, 120, and 240 mg ae/L for 6-48 h (short-term). Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), carboxylesterase (CbE), and glutathione S-transferase (GST) activities were measured among tadpoles sampled from those treatments that displayed survival rates >85%. Forty-eight-hour LC_{50} for *R. arenarum* tadpoles exposed to CF-GLY in the static tests ranged from ULT = 2.42 to FAV = 77.52 mg ae/L. For all CF-GLY, the LC50 values stabilized at 24 h of exposure. Tadpoles exposed to all CF-GLY concentrations at 48 h showed decreases in the activities of AChE (control = 17.50 ± 2.23 nmol/min/mg/protein; maximum inhibition INF 30 mg ae/L, 71.52%), BChE (control = 6.31 ± 0.86 nmol/min/mg/protein; maximum inhibition INF 15 mg ae/L, 78.84%), CbE (control = 4.39 ± 0.46 nmol/min/mg/protein; maximum inhibition INF 15 mg ae/L, 81.18%), and GST (control = 4.86 ± 0.49 nmol/min/mg/protein; maximum inhibition INF 1.87 mg ae/L, 86.12%). These results indicate that CF-GLY produce a wide range of toxicities and that all enzymatic parameters tested may be good early indicators of herbicide contamination in *R. arenarum* tadpoles.

Materials and methods

Experimental Design

Prometamorphic tadpoles of R. arenarum (larvae) were collected from temporary ponds in natural parafluvial forests in Argentina. All tadpoles were collected from non-agricultural areas. The author suggests therefore that they likely had minimal exposure to pesticides. The average total size (measured from snout to tail tip) was 0.93 ± 0.09 mm and weight was 0.27 ± 0.08 g; stages 36-38 (Gosner 1960). The tadpoles were acclimatized for 48 h to a 12:12-h light-to-dark cycle with dechlorinated tap water of pH 7.4 ± 0.05; conductivity 165 ± 12.5 µmhos/cm; dissolved oxygen concentration 6.5 ± 1.5 mg/L; and hardness 50.6 mg/L $CaCO_3$ at 22 ± 2 °C.

Short-term (6-, 12-, 24-, and 48-h) static tests were performed to evaluate the toxicity of CF-GLY: Roundup Ultra-Max (ULT) (commercial grade; 74.7% a.i.); Infosato (INF), Glifoglex (GLE), and C-K Yuyos FAV (FAV) (commercial grade; 48% a.i., respectively). All four GLY formulated products contain ammonium salt N-(phosphonomethyl) glycine and some unspecified surfactant, classified as an inert ingredient, that does not require testing or listing on the formulated product.

The glass tanks (12.5 cm diameter and 13.5 cm height) with 1 L DTW and 7 tadpoles/tank were used in the experiments. Animals were not fed during the toxicity tests. Tests were conducted at 22 ± 2 °C and under a 12:12-h light-to-dark photoperiod. The nominal concentrations of CF-GLY used were as follows: 1.85, 3.75, 7.5, 15, 30, 60, 120, and 240 mg ae/L. Negative controls with DTW were used. Both control and test solutions were made in triplicate. Treatments were randomly assigned to the tanks as was the order in which the tanks were sampled.

Enzymatic Determinations

Control and treated animals at all concentrations that had a survival rate >85% at 48 h were killed according to American Society of Ichthyologists and Herpetologists (2004) criteria. Whole tadpoles were homogenized (on ice) in 0.1% t-octylphenoxypolyethoxy ethanol (triton X-100) in 25 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a polytron. The homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant was collected (heretofore called ‘‘crude extract’’). Protein concentrations in the supernatants were determined according to the Biuret method (Kingbley 1942). When sample volume was enough, enzyme kinetics assays were performed in triplicate or duplicate. AChE and BChE activities were measured according to Ellman *et al.* (1961). CbE activity using α-naphthyl acetate (α-NA) was measured by the Gomori method (1953). GST activity was determined spectrophotometrically by the method described by Habig *et al.* (1974).

Statistical Analysis

Lethal concentration (LC_{50}) values and their respective 95% confidence limits (CLs) were determined by the Trimmed Spearman-Karber method (Hamilton *et al.* 1977). The LC_{50} estimates were subjected to one-way analysis of variance (ANOVA) followed by post hoc contrast with Duncan’s Multiple Range Test at $p < 0.05$. The data of enzymatic activity were expressed as the mean ± SEM. In all experiments, replicates were tested for differences using ANOVA (Hurlbert 1984). No significant differences were found among replicates ($p > 0.05$); thus, no tank effect was identified, and replicates were pooled. The influence of pesticide treatments and concentrations on each B-esterase and GST enzyme activities were analyzed with two-way ANOVA using general linear models (GLMs) and Dunnet test for post hoc comparisons. Data were tested for variance homogeneity and normality (Kolmogorov-Smirnov test and Levene test). Statistical analyses were performed using INFOSTAT/P® 1.1 for Windows software (Grupo InfoStat Professional, FCA, Universidad Nacional de Cordoba, Argentina). The criterion for significance was $\alpha = 0.05$.

Results

The LC_{50} values for the formulations tested are presented in Table 1.

ULT was the most toxic herbicide for assay tadpoles, followed by INF, GLE, and FAV. LC_{50} values at 8 h ranged from ULT=2.42 to LC_{50} FAV=77.52 mg ae/L. The LC_{50} values were stabilized at 24 h of exposure.

Table 1 (from Lajmanovich R. C. *et al.* 2011, AECT, Vol. 60, No. 4, pp. 681-9). Comparison of LC50 (95% CLs) (mg ae/L) values of *R. arenarum* tadpoles exposed at different CF-GLY: ULT, INF, GLE, and FAV.

Herbicide	6 h	12 h	24 h	48 h
ULT	5.62^{a} (4.07–7.15)	3.26^{a} (2.66–3.85)	2.42^{a} (2.19–2.65)	2.42^{a} (2.19–2.65)
INF	49.65^{b} (41.20–58.11)	47.25^{b} (38.61–55.89)	38.76^{b} (34.98–42.54)	38.76^{b} (34.98–42.54)
GLE	96.87^{c} (73.83–119.91)	77.52^{c} (70.14–84.90)	73.77^{c} (64.23–83.31)	73.77^{c} (64.23–83.31)
FAV	104.33^{c} (70.85–137.81)	84.06^{c} (75.24–92.88)	77.52^{c} (70.14–84.90)	77.52^{c} (70.14–84.90)

Note: Column means sharing a common letter are not significantly different at the 5% level of probability by Duncan’s multiple range test

The statistical analysis of the influence of pesticide treatments and concentrations on each B-esterase and GST enzyme activities were analyzed with two-way ANOVA using general linear models (GLMs) and Dunnet test for post hoc comparisons, is presented in Table 2.

Table 2 (from Lajmanovich R. C. *et al.* 2011, AECT, Vol. 60, No. 4, pp. 681-9). GLMs between B-sterases and GST enzyme activities and type and concentration of CF-GLY.

	AChE	BChE	CbE	GST
GLM	4.26***	4.90***	3.05***	4.26***
Treatment factor	13.2***	17.71***	10.79***	15.1***
Concentration factor	3.24**	2.13*	1.13 (NS)	0.59 (NS)
Treatment × concentration	1.80*	2.03*	1.49 (NS)	2.46**
R^2 squared (%)	41.9	47.4	34.2	42.5

NS = not significant

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

AChE and BChE activities, CbE activity and GST activity is presented below.

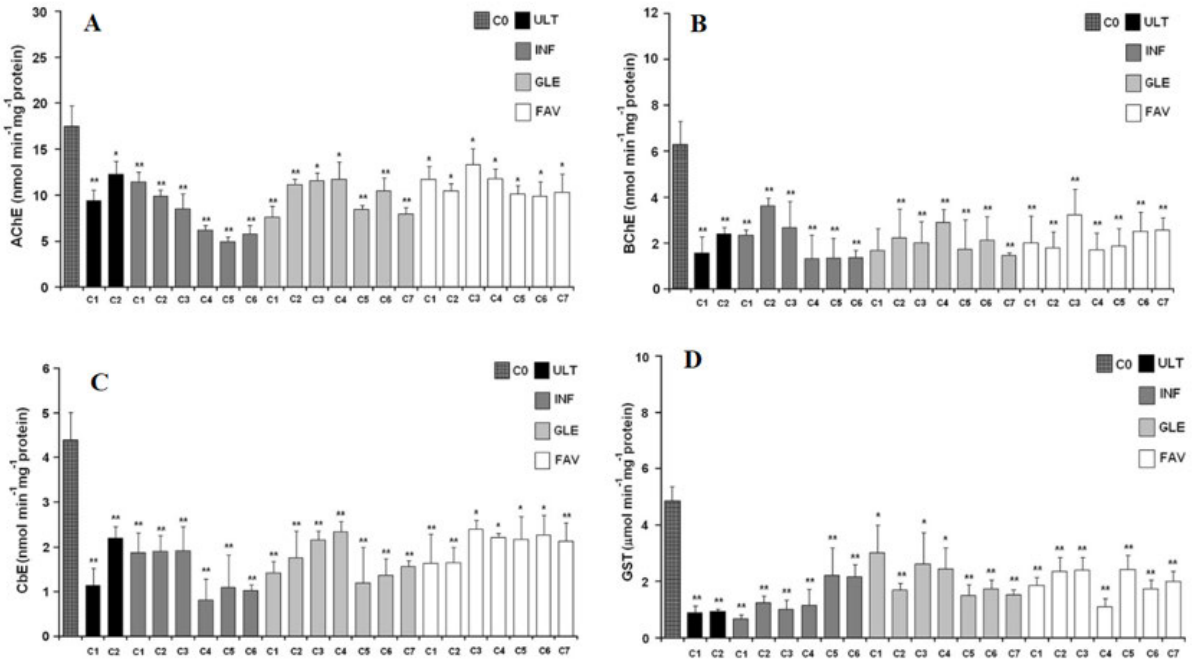


Figure 1 (from Lajmanovich R. C. *et al.* 2011, AECT, Vol. 60, No. 4, pp. 681-9). Effects of commercial GLY exposure (48-h): C0=Control, ULT, INF, GLE, and FAV on the activity of **A**) AChE, **B**) BChE, **C**) carboxylesterase (CbE), and **D**) GST in *R. arenarum* tadpoles. C1=1.85, C2=3.75, C3=7.5, C4=15, C5=30, C6=60, and C7=120 mg ae/L. Data are expressed as mean ± SEM, N=7-10. Significantly different from control (**p < 0.01;*p < 0.05; Dunnett's test).

In this study the authors highlighted novel insights regarding the side effects of a commercial herbicide formulation containing glyphosate on the biologic and biochemical responses of a common native anuran species. The results demonstrate the difficulty of formulating environmental regulations to legislate the CF-GLY, taking into account that different commercial formulations can produce widely different toxicities. The disparities likely occur because of the inclusion of unspecified surfactants, which are often referred to as ''inert'' or which remain proprietary information (i.e., ''trade secrets''). In this way, they suggest that those trade secrets should be informed ones because in general, farmers use these products to increase crop yields and often ignore herbicide toxicity to wild fauna. In addition, companies have expanded programs to include developing new formulations and/or marketing strategies to differentiate their product from competing CF-GLY products.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). LC_{50} were generated using very high concentrations tested. Enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological regulatory risk assessment. None of the tested formulations is the representative formulation for the glyphosate EU renewal. Therefore the article is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The four tested formulations are reported to have unspecified surfactants and it is thus not possible to conclude how representative they are for the glyphosate EU renewal. However, information on the surfactant used in one of the formulations – Roundup Ultra Max is available from a different publication (Wagner *et al.* 2017). Based on this information (ether amine ethoxylate as surfactant), the RMS considers the results on Roundup Ultra Max to be relevant. The results on Infosato, C-K YUYOS FAV and Glifoglex are considered less relevant but supplementary and will be used in a WoE approach.

This laboratory study generated 48h LC_{50} values ranging 2.42 – 77.52 mg a.e./L for *Rhinella arenarum* tadpoles.

The RMS considers this study to be reliable with restrictions, since no analytical confirmation of test concentrations was performed.

Data point	CA 9
Report author	Lajmanovich R. C. *et al.*
Report year	2013
Report title	Individual and Mixture Toxicity of Commercial Formulations Containing Glyphosate, Metsulfuron-Methyl, Bispyribac-Sodium, and Picloram on *Rhinella arenarum* Tadpoles
Document No.	Water Air and Soil Pollution (2013), Vol. 224, No. 3, pp. Article No.: 1404
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant;	Not relevant by full text

See also RMS analysis in RMS box

The effects of four commercial formulations of herbicides (glyphosate [GLY], metsulfuron-methyl [MET], bispyribac-sodium [BIS], and picloram [PIC]) individually, and in three 50:50 mixtures (GLY–MET, GLY–BIS, GLY–PIC) on the common toad *Rhinella arenarum* (Anura: Bufonidae) tadpoles. Enzymatic parameters such as, glutathione S-transferase (GST), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities, as well as erythrocyte nuclear abnormalities (ENA) were studied. Interactions between herbicides in mixtures were evaluated and classified as additive, synergistic, or antagonistic. Toxicity results (48-h LC_{50}) showed that PIC was the most toxic herbicide, followed by BIS, GLY, and MET, while GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. All commercial herbicide formulations and their mixtures significantly inhibited BChE activity in exposed tadpoles. The AChE activity was also inhibited by all herbicides and their mixtures, except by GLY–BIS. The inhibition of GST activity was only significant for GLY, MET, PIC, and GLY–MET.A significant increase in the frequency of ENA was found for tadpoles exposed either to commercial herbicide formulations or to mixtures, except for GLY. All the mixtures showed synergism for BChE activity while for AChE only the GLY–MET and GLY–PIC mixtures acted synergistically. GLY–MET showed synergism for GST, whereas for ENA, the mixture GLY–BIS was antagonistic. This study with *R. arenarum* tadpoles demonstrates that the interactions between three of the most intensively used herbicides in soybean crops results in synergistic effects on mortality and neurotoxicity and synergistic or additive effects in genotoxicity.

Materials and methods

Tadpoles of *Rinella arenarum* were selected as model test organisms. This common anuran has an extensive neotropical distribution, and it is frequently found in forest, wetlands, agricultural land, and urban territories. Its larvae exhibit aggregative behavior and have been recently characterized by their sensitivity to GLY. Premetamorphic larvae were collected in January 2011 from temporary ponds in natural floodplains of the Paraná River in Argentina.

For short-term (48-h) static toxicity tests, commercial formulations of GLY (74.7 % active ingredient [a.i.], N-(phosphonomethyl) glycine; Ultra-Max®], MET (60 % a.i., methyl 2-(4-methoxy-6-methyl-1,3,5- triazin-2-ylcarbamoylsulfamoyl) benzoate]; Metsulfuron 60®, BIS (40 % a.i., sodium 2,6-bis (4,6-dimethoxypyrimidin-2-yloxy) benzoate], and PIC (27.7 % a.i., 4-amino-3,5,6- trichloropicolinic acid, Tordon 24-K®]. The herbicides were tested as a complex commercial mixture because this is the form in which they are applied in cultivated fields and introduced into the environment.

Glass aquariums with 1 L of dechlorinated tap water and seven tadpoles per recipient with a loading ratio of approximately 0.38 (g/L) (N=336 per treatment) were used in the experiments. Tests were conducted at 22 ± 2 °C and at 12-h light/dark cycle. The nominal concentrations used to test single toxicities of four herbicides were: 0.0097, 0.0195, 0.039, 0.0781, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 mg a.i./L. The toxicity of the following mixtures GLY and MET, GLY and BIS, and GLY and PIC in 50:50 combinations were evaluated using the same nominal concentrations.

AChE and BChE measurements
Whole tadpoles were homogenized (on ice) in Triton X-100 in 25 mM tris (hydroxymethyl) aminomethane hydrochloride (pH 8.0) using a homogenizer. The homogenates were centrifuged at 14,000 rpm for 15 min at 4 °C, and the supernatant was collected. Total protein concentrations in the supernatants were determined according to the Biuret method. Enzyme kinetics assays were performed in triplicate. AChE and BChE activities were measured according to the Ellman et.al. method. GST activity was determined spectrophotometrically by the method described by Habig *et al.* as adapted by Habdous *et al.* for mammal serum GST activity. The enzyme assay was performed at 340 nm in 100 mM Na phosphate buffer (pH 6.5), 2 mM CDNB, and 5 mM GSH.

Gentoxicity testing

Blood was taken from each tadpole by cardiac puncture, blood smears were prepared on clean slides, fixed and stained by the May-Grunwald-Giemsa method. Genotoxicity was tested using the presence of erythrocyte nuclear abnormalities (ENA), and carried out in RBCs according to the procedures of Guilherme *et al.*.

Statistics
Lethal concentration (LC_{50}) values and their respective 95 % confidence intervals (CI) were determined by the Trimmed Spearman-Karber method (Hamilton *et al.*). The LC_{50} estimates were subjected to one-way analyses of variance (ANOVA) followed by post hoc contrast with Duncan's multiple range test. The mortality data were statistically evaluated by ANOVA using Dunnett's procedure for multiple comparisons in order to determine the NOEC and the lowest-observed effect concentration (LOEC).

Results

PIC was considered the most toxic herbicide for assay tadpoles within the study, followed by BIS, GLY, and MET. LC_{50} values at 48 h ranged from PIC=0.025 to LC_{50} MET=105.56 mg a.i/L. GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. LC_{50} values at 48 h ranged from GLY–PIC=0.051 to LC_{50} GLY–MET=25.62 mg a.i/L. According to Marking's additive index, all herbicide mixtures evaluated displayed synergistic toxicity, with a sum of activity of 2.04 to 2.18. Furthermore, the negative results of AI indicated low additivity. In the case of AChE, GLY–MET and GLY–PIC were synergistic; for BChE, all mixtures were synergistic; on the contrary, for GST only GLY–MET mixture was synergistic. For ENA, GLY–BIS was antagonistic; and in all other cases, the interactions were additive.

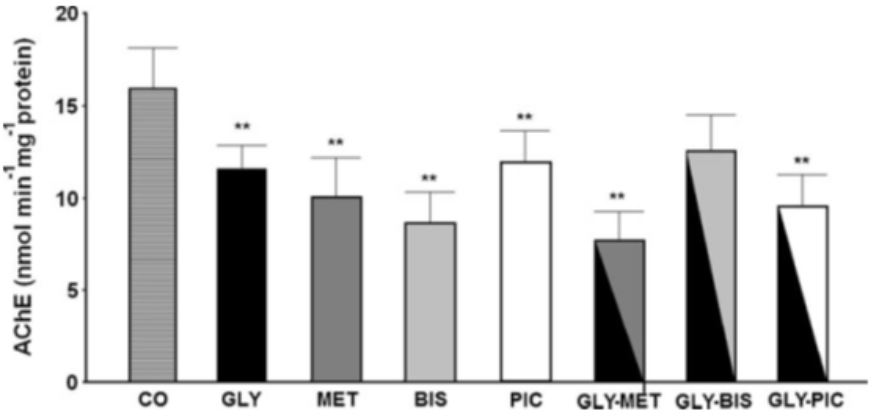


Figure 1 (from Lajmanovich R. C. *et al.* 2013, Water Air Soil Pollut, Vol. 224, No. 3). Effects of commercial herbicide exposure (48-h) at no observed effect concentration (NOEC) on the acetylcholinesterase (AChE) activity in *R. arenarum* tadpoles. C0 control, GLY glyphosate, MET metsulfuron-methyl, BIS bispyribac-sodium, PIC picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, N=7-10. Significantly different from control (**p < 0.01; Dunnett's test).

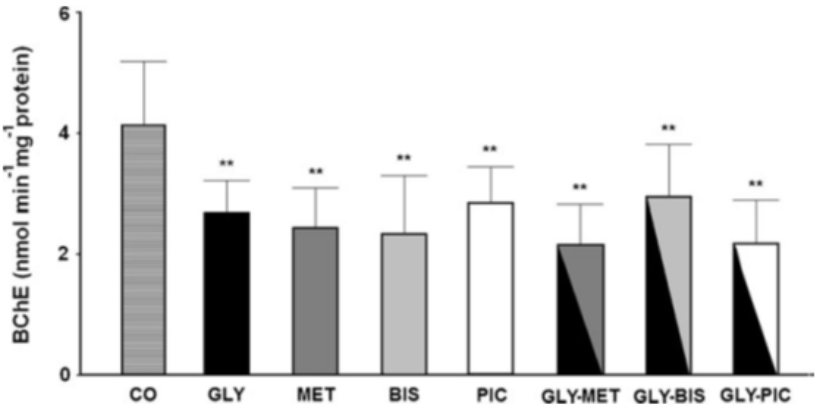


Figure 2 (from Lajmanovich R. C. *et al.* 2013, Water Air Soil Pollut, Vol. 224, No. 3). Effects of commercial herbicide exposure (48-h) at no observed effect concentration (NOEC) on the butyrylcholinesterase (BChE) activity in *R. arenarum* tadpoles. C0 control, GLY glyphosate, MET metsulfuron-methyl, BIS bispyribac-sodium, PIC picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, N=7-10. Significantly different from control (**p<0.01; Dunnett's test).

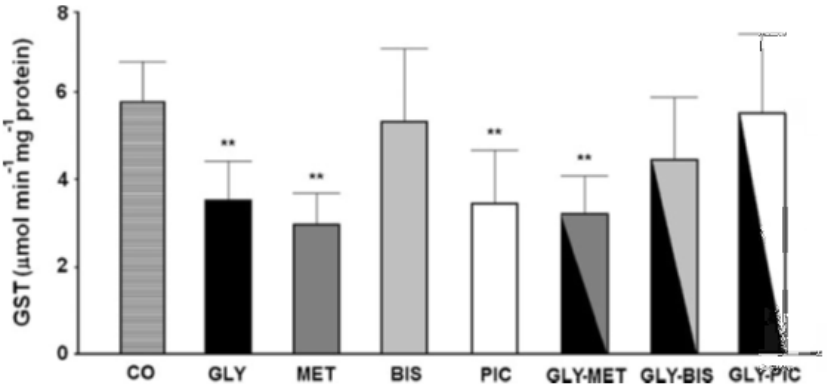


Figure 3 (from Lajmanovich R. C. *et al.* 2013, Water Air Soil Pollut, Vol. 224, No. 3). Effects of commercial herbicide exposure (48-h) at no observed effect concentration (NOEC) on the glutathione-S-transferase (GST) activity in *R. arenarum* tadpoles. C0 control, GLY glyphosate, MET metsulfuron-methyl, BIS bispyribacsodium, PIC picloram, and mixture of 50:50 GLY–MET, GLY–BIS, and GLY–PIC. Data are expressed as mean ± SEM, N=7-10. Significantly different from control (**p<0.01; Dunnett's test).

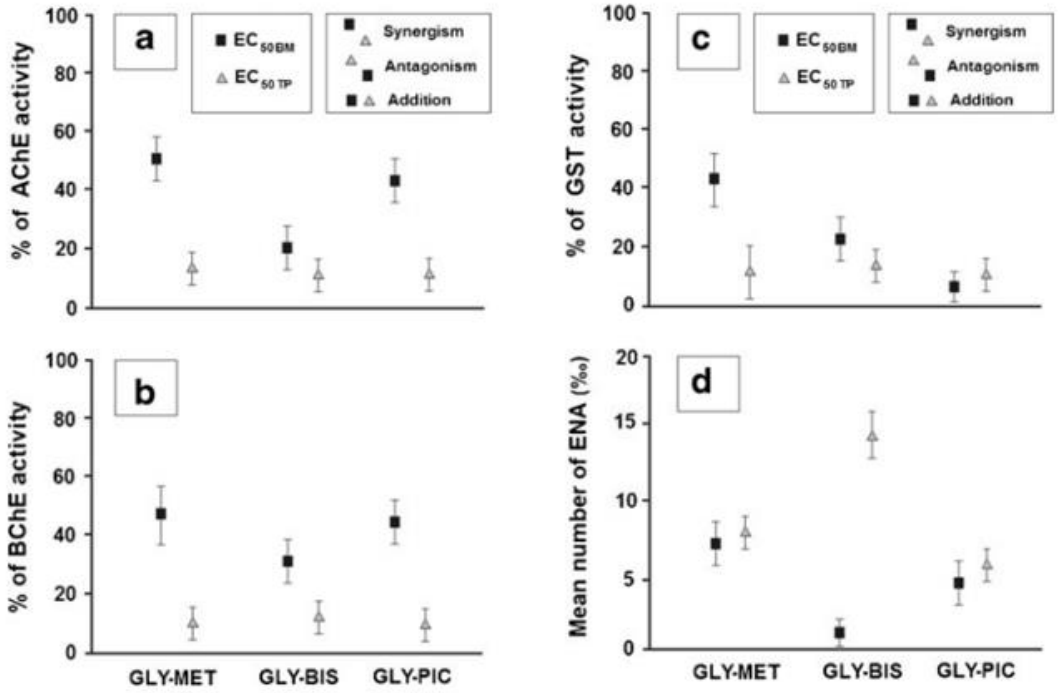


Figure 4 (from Lajmanovich R. C. *et al.* 2013, Water Air Soil Pollut, Vol. 224, No. 3). Effects of commercial herbicide exposure (48-h) on the percentages of acetylcholinesterase (AChE) (a), butyrylcholinesterase (BChE) (b), glutathione-S-transferase (GST) (c) inhibitions, and mean number of frequency of erythrocyte nuclear abnormalities (ENA) (d) in *R. arenarum* tadpoles. Median effective concentration calculated in binary mixture (EC_{50} BM) and median effective concentration (toxic potential) (EC50 TP). GLY glyphosate, MET metsulfuron-methyl, BIS bispyribac-sodium, PIC picloram in 50:50 mixture. Each point represents the mean (N=7-10), and error bars indicate the 95 % CIs of the mean.

Overall the author state that the results indicate that PIC was the most toxic herbicide for *R. arenarum* tadpoles, followed by BIS, GLY, and MET, while GLY–PIC was the most toxic mixture, followed by GLY–BIS, and GLY–MET. Also, all commercial herbicides tested and their mixtures in sublethal concentrations (NOEC) inhibit ChEs activity and induce genotoxicity in tadpoles exposed. Finally, *R.*

arenarum tadpoles exposed to mixtures of the most intensively used herbicides in crops of soybean crops, showed mortality synergy, either concentration-synergistic or additive neurotoxicity and genotoxicity. This implies that single-chemical assessments will systematically underestimate the actual risks to amphibian species in waterbodies where mixtures of herbicides potentially occur.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). Observations is caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity). Enzyme, cellular and molecular level endpoints are discussed that are difficult to relate to the EU level ecotoxicological regulatory risk assessment.

In addition, the glyphosate formulation tested (74.7 % active ingredient; Ultra-Max®, Monsanto Co., Argentina) is not the representative formulation for the glyphosate EU renewal. Therefore, the article is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning for non-relevance of the study. Information on the surfactant used in one of the formulations – Roundup Ultra Max is available from a different publication (Wagner *et al.* 2017). Based on this information (ether amine ethoxylate as surfactant), the RMS considers the results on Roundup Ultra Max to be relevant.

Moreover, the factors listed to justify the difficulty of extrapolating the results to the EU are not relevant, since this is a laboratory study. The fact that all test organisms were collected from the same ponds means that any potential background exposure to glyphosate (or other stressors) is also accounted for by the control organisms. In addition, the statement that the observations are caused by mixtures and cannot be attributed to the substance of concern is inaccurate, since also individual compounds were investigated, although these results were not included in the study summary by the applicant.

Exposure of *Rinella arenarum* tadpoles to Ultra-Max® resulted in 48 h LC_{50}= 13.20 mg a.i./L (95 % CI 11.57–15.05 mg a.i./L), corresponding to 9.9 a.e. mg/L.

The RMS considers this study to be reliable with restrictions, since no analytical confirmation of test concentrations was performed.

Data point	CA 9
Report author	Rissoli Zanelli R. *et al.*
Report year	2016

Report title	Effects of glyphosate and the glyphosate based herbicides Roundup Original® and Roundup Transorb® on respiratory morphophysiology of bullfrog tadpoles
Document source	Chemosphere, (2016) Vol. 156, pp. 37 44
Short description of literature article	Amphibian tadpoles often experience variations in O_2 availability in their aquatic habitats; an ability to tolerate hypoxia can condition their survival and fitness. The article aimed to evaluate the impacts of sublethal concentrations of glyphosate (1 mg/L), Roundup Original (1 mg/L glyphosate a. s.) and Roundup Transorb® (1 mg/L glyphosate a. s) on metabolic rate ($V \cdot O_2$ mLO_2 Kg1/h) of bullfrog tadpoles during normoxia and graded hypoxia, and related this to morphology of their skin, their major site of gas exchange.
Short description of findings	In control (CT) V O_2 remained unaltered from normoxia until 40 mmHg, indicating a critical O_2 tension between 40 and 20 mmHg. Glyphosate significantly reduced V O_2, possibly due to epidermal hypertrophy, which increased O_2 diffusion distance to O_2 uptake. In contrast, Roundup Transorb® increased V O_2 during hypoxia, indicating an influence of "inert" compounds and surfactants. V O_2 of Roundup Original did not differ from CT, suggesting that any increase in V O_2 caused by exposure was antagonized by epidermal hypertrophy. All tested items caused marked alterations in skin morphology, with cell and epithelium wall presenting hyperplasia or hypertrophy and chromatid rupture. In summary, glyphosate, Roundup Original and Roundup Transorb® exert different effects in bullfrog tadpoles, in particular the surfactants and inert compounds appear to influence oxygen uptake.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). Endpoints presented are not relevant to the EU regulatory ecotoxicological risk assessment. In addition, the tested formulations are not the representative formulation for the glyphosate EU renewal. **Further points of clarification:** Although Roundup formulations were tested, they do however contain different surfactant system and composition than the representative formulation MON 52276 for the glyphosate EU renewal. The surfactant system used in the formulation Roundup Original is polyethoxylene amine based (POEA), whereas the surfactant used in MON 52276 is quaternary ammonium based. POEA surfactant is not permitted in formulated herbicidal products in the EU. Roundup Transorb® contains 540 g/L and glyphosate in a form of a potassium salt, whereas MON 52276 has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L and is formulated using glyphosate isopropyl ammonium salt. As the performance / efficacy of herbicidal formulations is dependent on the surfactant system / co formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal.

RMS comments and conclusion	The RMS agrees that the results regarding Roundup Original® are not relevant for this risk assessment, as the formulation includes a surfactant of known high toxicity, which is similar to substances not allowed in the EU (Regulation (EU) 2016/1313 and/or Regulation (EU) 2021/383. Although Roundup Transorb® contains unknown surfactants according to this paper, another publication on fish (Rocha et al. 2015) suggests that POEA is one of the surfactants included in this formulation. Hence, the RMS considers the results on Roundup Transorb® to be not relevant. Furthermore, the results on the active substance glyphosate are relevant and will be considered. Glyphosate caused morphologic alteration of epidermis (increased thickness and some chromatid fragmentation) as well as a reduction in O_2 uptake during hypoxia. Such morphological effects, as well as the of impairments to systemic uptake or distribution of O_2 can be considered as population- relevant and therefore useful for the risk assessment. The study authors proposed that the increased thickness of epidermis might be a response to avoid systemic absorption of glyphosate. NOEC could not be determined, as only one concentration was tested, and it resulted in significant effects on both O_2 uptake and epidermis morphology. LOEC for glyphosate is thus 1 mg a.e./L. The RMS considers this study to be reliable with restrictions since no analytical confirmation of the test concentration was performed.

Data point	CA 9
Report author	Ruuskanen S. *et al.*
Report year	2020
Report title	Female Preference and Adverse Developmental Effects of Glyphosate-Based Herbicides on Ecologically Relevant Traits in Japanese Quails
Document No.	Environmental science & technology (2020), Vol. 54, No. 2, pp. 1128-1135
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The preference or avoidance behavior of Japanese quails (*Coturnix japonica*) to glyphosate-based herbicide (GBH) Roundup Flex contaminated food was established. The current study demonstrated that females preferred GBH contaminated food compared to control food. In females, exposure to GBHs caused delayed plumage development, and GBH residues were present in eggs, muscles, and liver. These

results indicate that female preference is not adaptive, potentially exposing nontarget animals to greater risk of adverse effects of GBHs in natural and agricultural environments.

Materials and methods

Thirty six '1 year old' Japanese quail were paired up (male + female) and transferred to cages (1 m × 1 m, height 0.5 m, +20 °C and 16:8 photoperiod, with no visual contact to other birds) to acclimatize them to the cages. The birds were provided with organic food (the same food that was used in the subsequent trials, see below) during the acclimatization period, in the middle of the cage. Food, water, lime, and grit were available ad libitum, and a wooden box provided shelter in the test cages. The feeders used during the GBH preference/avoidance experiment were the same as those used during regular feeding. The researchers performed a two-choice experiment where each bird was provided food contaminated with glyphosate-based herbicides (hereafter, GBH food) and control food simultaneously and tested repeatedly. The GBH food consisted of organic food (organic food for laying poultry, "Luonnon Punaheltta" Danish Agro, Denmark) with the added commercial GBH, Roundup Flex (480 g/L glyphosate, present as 588 g/L [43.8% w/w] of potassium salt of glyphosate, with surfactants alkylpolyglycoside (5% of weight) and nitrotryl (1% of weight), AXGD42311 5/7/2017, Monsanto), corresponding to 20 mg of glyphosate/ kg food. This dose corresponds to the upper permitted limit for glyphosate residues in cereals for human consumption in the EU19 and expected glyphosate residue dietary burden in chickens (23 mg/kg). The GBH food was prepared by adding diluted GBHs to organic food, after which the food was allowed to dry and crushed to a suitable grain size for quails. Because of its low volatility, glyphosate remains in the food pellets during the drying. The control food was prepared similarly but the GBH was replaced with tap water and was analysed for glyphosate residues. Given that the glyphosate dose (20 mg/kg) was within the recommended limits (for humans), no adverse effects of treated food was expected in this short-term trial. In the experimental design, each quail was provided one feeder with GBH food and one feeder with control food simultaneously. The feeders were located at opposite sides of the test cage. In order to avoid lateralization effects, the feeder with GBH food was placed randomly on the right or left side, alternating between individuals. The quails were food deprived for 1 h prior to the trials in order to stimulate their appetite. Video cameras were placed above the cages 1 day prior to the test to acclimatize the birds to the equipment. The behavior of the quails was video recorded for 30 min after providing the GBH and control feeders. One 30 min trial was performed for each quail per day. The trial was repeated four times over consecutive days for each bird (i.e., four trials per individual). The GBH and control food were located at the same positions during the four trials for each focal bird. Altogether, 144 thirty-minute trials were performed (36 individuals, four trials per individual). All trials were performed between 9 and 12 p.m., and the order of the tests across individuals was rotated each day to minimize any effects of time of the day. After the four trials, the researchers measured the individual consumption of the GBH and control foods over 20 h, by providing 20 g of GBHs and control food in the regular feeders at 4 p.m. of the 4th trial day, and weighed the remains of the food on the following day at 12 p.m. to estimate consumption.

One person blind scored the following aspects based on videos from each of the 30 min trials: (1) the first choice, that is, which feeder (GBH or control) the bird visited first, (2) the total time spent close to each feeder, that is, the time spent to inspect the feeder, and (3) the total number of pecks to each feeder.

Results

The average glyphosate concentration in three pools (4−6 individuals in each pool) of excreta samples (urine and fecal matter combined) was 199 mg/kg (standard error 10.5 mg/kg), see details in Table 1.

Table 1 (from Ruuskanen S. *et al.* 2020, ES&T, Vol. 54, No. 2, pp. 1128-1135). Glyphosate Residues (mg/kg Tissue, Average, and Standard Deviation) in Various Tissues and Excreta (Fecaland Urine Combined), Separately for Females and Males.[a]

tissue type	treatment	sex	exposure (months)	average glyphosate (mg/kg)	SD
egg	GBH	female	10	0.76	0.16
liver	GBH	male	5	0.74	0.50
liver	GBH	male	12	1.33	0.21
liver	GBH	female	12	4.10	1.10
muscle	GBH	male	12	0.10	0.02
muscle	GBH	female	12	0.24	0.09
excreta	GBH	male	12	209.05	8.40
excreta	GBH	female	12	179.50	NA

[a]Samples from several, randomly chosen animals have been pooled (see the text for details on sample sizes).

Concerning peck activity at the GBH feeders, these results are presented in Fig. 1.

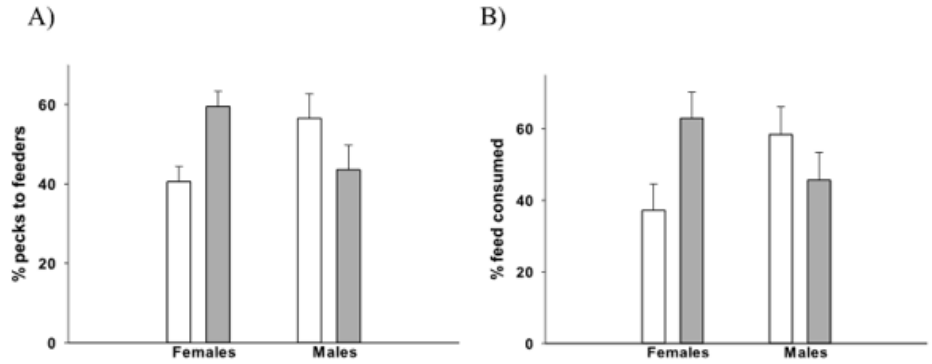


Figure 1 (from Ruuskanen S. *et al.* 2020, ES&T, Vol. 54, No. 2, pp. 1128-1135). (A) Percentage (SE) of the number of pecks at the GBH feeder (gray bar) and control feeder (white bar) during 30 min trials in Japanese quails. Only trials where the focal individual visited both the GBH and control feeder are included. N= 42 trials (19 individuals) for females, and 16 trials (8 individuals) for males. (B) Percentage (SE) of GBH-contaminated food (gray bars) and control food (white bars) consumed by Japanese quails in overnight trials. N = 19 females and 17 males.

The effects on growth of male and female birds are presented in Table 2.

Table 2 (from Ruuskanen S. *et al.* 2020, ES&T, Vol. 54, No. 2, pp. 1128-1135). Growth in Relation to Dietary GBH Exposure in Japanese Quails[a]

		linear growth (slope)		body mass (g) at maturity		body mass (g) at 12 months	
		mean (SE)	*N*	mean (SE)	*N*	mean (SE)	*N*
females	GBH	3.19 (0.13)	15	174.8 (7.1)	14	181.3 (6.8)	13
	CO	3.25 (0.15)	14	182.7 (6.8)	13	194.6 (7.3)	13
males	GBH	2.98 (0.09)	23	146.1 (4.1)	23	161.1 (8.3)	13
	CO	3.08 (0.09)	23	148.3 (4.5)	23	174.8 (7.7)	13

[a]The means (SE) of linear growth, body mass at maturity, and body mass at 12 months of exposure in GBH exposure are reported, separately for female and males. GBH = glyphosate-based herbicide treatment, CO = control treatment. See the text for statistics.

The effects on feather plumage are presented in Fig. 2.

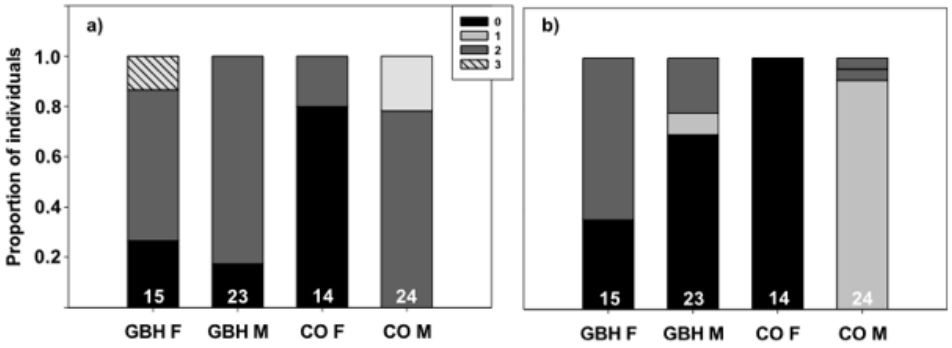


Figure 2 (from Ruuskanen S. *et al.* 2020, ES&T, Vol. 54, No. 2, pp. 1128-1135). Body feather plumage development in relation to dietary GBH exposure in Japanese quails. Proportions of individuals in different plumage development categories (0 = normal development, 1 = slightly delayed development, some stubs of body feathers, 2 = delayed development, some bare skin visible at the backside, and 3 = severely delayed development, multiple patches of bare skin areas) are shown at the age of 5 weeks (A) and 9 weeks (B). GBH = GBH treatment, CO = control treatment, F = female, M = male. Numbers in the bars refer to sample sizes in each category. See the text for statistics.

The authors indicated that they have provided novel data and a wider perspective on the behavioral and developmental effects associated with GBHs in and on the environment, which is critical information for the EU assessment of the renewal of the authorization for GBH use. They state that the results add to the increasing pool of studies on the adverse effects of GBHs on developmental and physiological responses in a variety of nontarget vertebrate and invertebrate taxa. They propose that in natural and agricultural environments, a preference for GBHs may affect the spatial distribution patterns or habitat choice of the organisms, while potential additional adverse fitness effects may have consequences at the population level. Finally, they suggest that glyphosate accumulation in the breast muscles, liver tissue, and eggs raises concerns on the transfer of GBHs in the food chain, as well as transgenerational effects, which should be further addressed.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested (Roundup Flex, 480 g/L glyphosate, present as 588 g/L [43.8% w/w] of potassium salt of glyphosate, with surfactants alkylpolyglycoside (5% of weight) and nitrotryl (1% of weight)) is not the representative formulation for the glyphosate EU renewal, thus the article is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

As the formulation Roundup Flex does not contain substances forbidden within the EU, the RMS considers the results based on this formulation to be relevant for the risk assessment.

This study showed that glyphosate residues were present in quail muscles and liver, as well as in eggs from exposed parents (average concentrations 0.17, 2.05 and 0.76 mg/kg, respectively) following chronic exposure to food contaminated with glyphosate-based herbicide (164 mg glyphosate/kg food). In addition, exposure to this contaminated diet led to reduced flight feather moult in female quails and delayed plumage development in juveniles, regardless of sex and age.

A short-term test with adult quails indicated that females preferred glyphosate-contaminated food (20 mg glyphosate/kg food) over control food. Based on all these findings, the RMS considers this study relevant for the risk assessment. Moreover, the RMS considers the study to be reliable.

Based on the feed intake estimated from Fig 1B and the adult body mass in the controls (Table 2), the daily glyphosate intake for female quails is estimated at 1.6 mg/kg bodyweight/day.

No NOEC could be determined from this study, as only one concentration was tested per experiment. Therefore, 20 h LOEC for food consumption by females and 8 weeks LOEC for plumage development are both 164 mg a.s/kg food.

It is noted that the effect levels in this study are more adverse than those from standard studies, which may need further consideration. In addition, the results are potentially relevant for assessment of ED, as feather development is regulated by hormones. More in-depth investigations of the mechanisms behind delayed plumage development/moult are needed.

Data point	CA 9
Report author	Wagner N. *et al.*
Report year	2017
Report title	Effects of a commonly used glyphosate-based herbicide formulation on early developmental stages of two anuran species
Document No.	Environmental science and pollution research international (2017), Vol. 24, No. 2, pp. 1495-1508
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The acute toxic effects (mortality, growth, and morphological changes) of the commonly used glyphosate-based herbicide formulation Roundup® UltraMax was studied on early aquatic developmental stages of two anuran species with different larval types (obligate vs. facultative filtrating suspension feeders), the African clawed frog (*Xenopus laevis*) and the Mediterranean painted frog (*Discoglossus pictus*). While *X. laevis* is an established anuran model organism in amphibian toxicological studies, the aim was to establish *D. pictus* as another model for species with facultative filtrating larvae. A special focus of the present study lies on malformations in *X. laevis* embryos, which were investigated using histological preparations. In general, embryos and larvae of *X. laevis* reacted more sensitive concerning lethal effects compared to early developmental stages of *D. pictus*. It was suggested that especially the different morphology of their filter apparatus and the higher volume of water pumped through the buccopharynx of *X. laevis* larvae lead to higher exposure to the formulation. The test substance induced similar lethal effects in *D. pictus* larvae as it does in the teleost standard test organism used in pesticide approval, the rainbow trout (*Oncorhynchus mykiss*), whereas embryos of both species are apparently more tolerant and, conversely, *X. laevis* larvae about two times more sensitive. In both species, early larvae always reacted significantly more sensitive than embryos. Exposure to the test substance increased malformation rates in embryos of both species in a concentration dependent manner, but not at environmentally relevant concentrations. However, the

assumed field safety, based on calculated surface water concentrations of the active ingredient (glyphosate), should be validated with realistic field data and buffer strips have to be urgently regarded to any aquatic amphibian habitat.

Materials and methods

Test organisms and test substance
The African clawed frog (*Xenopus laevis*) is a member of the anuran family Pipidae and originates from the southern part of Africa. Reproduction was initiated by injection of human chorionic gonadotropin into the dorsal lymph sac of both genders. The Mediterranean painted frog (*Discoglossus pictus*) inhabits a variety of aquatic and terrestrial habitats including cultivated landscapes. The eggs of *D. pictus* are available over the whole year which is not the case in, for instance, Central European species. To obtain eggs from *D. pictus*, females and males with visible nuptial pads at the forelimbs were placed (from terraria with an aquatic and a terrestrial part and ca. 23 °C) into small plastic terraria containing about 1 L of ca. 15 °C cold water over night. Aquatic life stages are relatively fast developing (in the closely related *D. scovazzi* about 1 month at ca. 23 °C from egg deposition until completed metamorphosis. These are benefits for its use in laboratory work. The effects of the commonly used glyphosate based herbicide Roundup® UltraMax (RU-UM) were studied on early developmental stages of both anuran organisms. According to its safety data sheet, RU-UM contains about 51 wt% of glyphosate isopropylamine salt (CAS 38641-94-0) as active ingredient (a.i.), which corresponds to 450 g a.i./L. The added surfactant (about 7.5 wt%) is not POEA but ether amine ethoxylate (CAS 71486-88-9). RU-UM is a non-selective, broad-spectrum foliar herbicide and, for instance, has been approved for 10 years in Germany (2/17/2004 to 12/31/2014, to use up 6/30/2016).

Test procedures
All experiments were conducted in a climate chamber at 23 ± 1 °C and 12:12-h light–dark cycle. All test solutions were freshly prepared with FETAX (Frog Embryo Teratogenesis Assay-Xenopus) solution. Ammonium, nitrate, nitrite, dissolved oxygen (all mg/L), and pH were measured at the beginning and end of the experiments. For quality assurance, water samples for glyphosate analysis of stock solution and each test concentration were taken and stored at -20 °C in stainless steel containers. Samples were shipped on ice to an external, DIN-certified laboratory (Eurofins SOFIA GmbH, Berlin) for liquid chromatography-mass spectrometry (LC-MS/MS). *X. laevis* embryo tests started with normally developed eggs at NF (Nieuwkoop and Faber 1956) stages 8–11, likewise *D. pictus* embryo tests with eggs at Gosner (Gosner 1960) stages 8–9 (=blastula to early gastrula). Glass petri dishes (60 mm in diameter) contained 10-mL test solution and 25 embryos. Experiments were terminated after 96 h when *X. laevis* individuals had reached NF stage 46 (*D. pictus* Gosner stage 22–23). The FETAX protocol (ASTM 1998) was applied, i.e., four controls were used, other test concentrations were duplicated (i.e., a total of 350 embryos), and solutions were renewed every 24 h (static renewal).

The FETAX protocol was slightly modified regarding the jelly coating of the eggs and positive controls. Jelly coats of eggs were not removed because of concerns that the dejelling L-cysteine would induce teratogenic effects and to study a more natural development. On the one hand, leaving the jelly coat on could have changed the toxicity seen compared to the studies that used L-cysteine; on the other hand, it was found that the jelly coat did not totally protect the embryo from xenobiotics. Furthermore, no positive control was used to prohibit cross-contamination with 6-aminonicotinamide in the climate chamber and to reduce the amount of test animals. Based on prior range finding tests (unpublished data), nominal concentrations for *X. lae*vis embryo tests were 0, 4, 9, 18, 36, 45, and 90 mg a.i./L and 0, 45, 90, 135, 180, and 225 mg a.i./L for *D. pictus* embryo tests.

Three malformed individuals and two control animals for pathohistological investigation were selected at the end of the *X. laevis* experiment. For the tests with early larvae, embryos were hatched in petri dishes (94 mm in diameter) containing 25 mL FETAX solution and 25 embryos each. In accordance with the standard protocol of the ASTM (2002), the tests with larvae started at NF stage 47 (*X. laevis*) and Gosner stage 25 (*D. pictus*), respectively (= free-swimming larvae). Only non-malformed larvae with normal swimming and feeding behavior were introduced. The larvae tests were performed using 5-L full glass aquaria containing 1 L of test solution and 10 larvae each (which were randomly assigned

to the experiment). Test concentrations were triplicated (i.e., a total of 180 larvae were tested). The non-renewal trials were terminated after 96 h when the larvae had reached NF stage 48 (*X. laevis*) and Gosner stage 26 (*D. pictus*). Based on prior range finding tests, nominal concentrations for *X. laevis* larvae tests were 0, 0.9, 1.8, 3.6, 4.5, and 9 mg a.i./L and 0, 4.5, 9, 18, 27, and 36 mg a.i./L for *D. pictus* larvae.

Histology and staining of cartilage
Embryos selected for clearing and staining were double stained for bone and cartilage. Specimens selected for serial sections were dehydrated, embedded in Histoplast S (Serva GmbH), and serially sectioned transversely at 7 µm using a Microm HM355 microtome. Sections were stained with azocarmine-red and anilin-blue (AZAN). Photographs of cleared and stained specimens were taken with a Zeiss SteREO Discovery V12 equipped with a Zeiss AxioCam Icc 1 digital camera. All morphological changes including malformations were identified according to the tables of Bantle *et al.* (1998).

Considered endpoints and statistical analysis
Mortality, malformations, and growth inhibition were monitored after 96 h. Embryos and larvae were photographed after euthanization with 150 to 200 mg/L MS-222 (OECD 2009) and fixation in 5 % formalin. The software BImageJ (National Institute of Health) was used to measure head–tail-length (or total length) (HTL) in embryos and larvae. Ninety-six-hour LC50 and ninety-six-hour EC50 (malformation) values (median lethal and effect concentration, respectively) were calculated with probit analyses. Significant differences were examined by overlap tests of 95% confidence intervals. Differences in mortality and malformation rates, and HTL (indicating growth inhibition) between groups were checked using one-way ANOVA (some data had to be Box-Cox transformed prior analysis), followed by Bonferroni corrected post hoc tests (for small sample sizes). If normal distribution and homogeneity of variances could not be reached by data transformation, Kruskal-Wallis rank sum test followed by Wilcoxon rank sum test with continuity corrections were conducted. The software R and the package MASS were applied for statistical analyses (R Developmental Core Team).

Results

Water analysis
In embryo tests, ammonium, nitrate, and nitrite were not measurable (i.e., 0 mg/L) at beginning and end of experiments. Dissolved oxygen and pH level remained constant through the experiments (8 and 6.8 mg/L, respectively). In the larvae tests, water parameters at beginning of the experiments (fresh test solutions) were the same as for embryo tests. At the end of the *X. laevis* tests (after 96 h), average values for ammonium were 0.4 mg/L, for nitrate 6.67 mg/L, and for dissolved oxygen 7 mg/L. At the end of the *D. pictus* tests, average values for ammonium were 0.4 mg/L, for nitrate 3.33 mg/L, and for dissolved oxygen 7 mg/L. At termination of both larvae experiments, nitrite was not measurable (0 mg/L) and pH level did not change (6.8). The slightly increase in ammonium and nitrate concentrations and decrease of dissolved oxygen were most probably caused by the increased metabolism of the larvae producing feces and by amounts of unconsumed food. Missing mortality demonstrated the validity of the study. Glyphosate concentration of the measured stock solution was confirmed by the analysis of the DIN-certified laboratory (450 mg a.i./L). Due to strong deviation, the fifth test concentration in *D. pictus* larvae test (27 mg a.i./L) was excluded from further analysis. Remaining measured test concentrations were in average 100.49 ± 3.07 % of the calculated concentrations and did not significantly differ (Wilcoxon-Mann-Whitney-Test: $W = 182$, $P = 0.98$).

Acute toxic effects of RU-UM on embryos
Because lack of overlap of the 95 % confidence intervals, *X. laevis* embryos were significantly more sensitive than *D. pictus* embryos; about fivefold more sensitive regarding lethal effects and about 3.5-fold more sensitive regarding the induction of malformations. Because already overlap tests of the 83–84 % confidence intervals seem to be enough to give an approximate $\alpha = 0.05$ test (Payton *et al.* 2003), the comparisons can be even seen as conservative results. Starting at 36 mg a.i./L, mortality significantly increased in *X. laevis* embryos ($F_{1,14} = 44.75$, $P < 0.001$) and starting at 180 mg a.i./L in *D. pictus* embryos ($F_{1,12} = 42.99$, $P < 0.001$). NOEC for mortality in *X. laevis* embryos was 18 mg a.i./L, but 135 mg a.i./L in *D. pictus* embryos. Congenital malformations occurred in the control group of *X. laevis* and *D. pictus*. They comprised edema (n = 6) and one time a combined malformation (axial and edema) in

X. laevis and axial (n = 4) and edema (n = 1) in *D. pictus*. The findings were understood as the basic spontaneous incidence. Incidence of malformations increased concentration dependently in *X. laevis* ($F_{1,9}$ = 24.77, P < 0.001). Also, in *D. pictus*, incidence of malformations increased with the concentration (49, 90, and 135 mg a.i./L, $F_{1,7}$ = 8.98, P < 0.05). However, for both embryo experiments, post hoc tests were not possible due to low survival in the replicates exposed to the highest test concentration with surviving embryos. Hence, no NOEC values can be stated, but for both species, Teratogenic Indices <1.5 indicate low teratogenic potential of the herbicide formulation. The goal of the Teratogenic Index (TI = LC_{50} values divided by the EC_{50} values) is to separate the lethal action of a compound from his teratogenic potential. TI values <1.5 should indicate low teratogenic potential of a substance because little or no separation exists between lethal and malformation inducing concentrations.

To describe the morphological changes after 96-h exposure of *X. laevis*, out of 16 fixed malformed embryos from the 36 mg a.i./L concentration group, three were selected for pathological investigations, and additionally, for comparison to individuals from the control. Changed proportions of body and tail, further the curved tail axis, the puffy body, and the small intestines are typical traits. They forecast internal deviations. At histopathological examination, edema was confirmed in the puffy body parts. Staining of cartilage revealed developmental retardation and deformation of elements of the viscero cranial skeleton. The most profound findings were the rather small and poorly differentiated infrarostralia. In addition, Meckel's cartilages (lower jaw), in ventral view, were more concave as opposed to having a convex shape in the control. The ceratohyalia of the hyoid arch and the ceratobranchial arches 1 and 2 were narrower compared to the control. Most cartilages seemed furthermore less developed and showed more irregular outlines. Additionally, the otic capsules were only indicated by slight chondrification in the mesenchymal condensations. No chondromalacia or other histopathological changes of the cartilage were seen. They comprised in the control group 7 embryos out of 96 surviving embryos (7 %) demonstrating the spontaneous incidence. In the 4.5 mg a.i./L dose group, 6 out of 44 surviving embryos (14 %) showed edema (n = 5) and one embryo a combination of edema and head malformations. In the 9 mg a.i./L dose group in one out of 41 surviving embryos (2 %), edema was seen. In the 18 mg a.i./L dose group in 9 out of 40 surviving embryos (23 %) head malformations (n = 3) and in four embryos, a combination of head malformation and edema in the lateral and ventral of the head-pharynx region were diagnosed. In each one, a combination of axial and head malformation and axial malformation and edema was recorded. In the 36 mg a.i./L dose group, all 16 surviving embryos (100 %) had a combination of head malformation and edema in the lateral and ventral of the head-pharynx region. It has to be underlined that these combinations of head malformation and edema in the lateral and ventral of the head-pharynx region were observed in the two highest test concentrations which did not induce 100% mortality (i.e., 18 and 36 mg a.i./L). The morphological changes in the *D. pictus* embryos comprised in the control group 5 out of 100 surviving embryos (5 %) demonstrating the spontaneous incidence. In the 45 mg a.i./L dose group, 6 out of 49 surviving embryos (12 %) had axial malformations (n = 5) and one embryo edema. In the 90 mg a.i./L dose group in 5 out of 48 surviving embryos (10 %), a combination of axial and head malformations (n = 4) and in one a singular axial malformation was diagnosed. In the 135 mg a.i./L dose group, 9 out of 20 surviving embryos (45 %) showed malformations, which included head malformations (n = 3), combinations of edema and head malformations (n = 2) and combinations of head and eye malformations (n = 4). Similar to increased mortality rates, the minimum concentration that inhibits growth (MCIG) in *X. laevis* embryos was 36 mg a.i./L and the NOEC for growth inhibition 18 mg a.i./L ($F_{1,228}$ = 9.61, P < 0.001). In *D. pictus*, MCIG was 136 mg a.i./L and consequently, the NOEC 90 mg a.i./L ($F_{1,215}$ = 27.72, P < 0.001).

Table 1 (from Wagner N. *et al.* 2017, ESPR, Vol. 24, No. 2, pp. 1495-1508). Lethal and teratogenic median concentrations of Roundup® UltraMax on embryos and early larvae of *X. laevis* and *D. pictus*. All values are given in mg a.i./L and were calculated using probit analyses with 95 % confidence limits stated.

Species	Developmental stage	96-h LC50	96-h EC50	TI
X. laevis	Embryos	25.82 (22.94; 28.70)	37.35 (28.42; 46.28)	0.69
D. pictus	Embryos	128.20 (121.51; 134.88)	173.24 (114.62; 231.89)	0.74
X. laevis	Early larvae	7.04 (6.24; 7.84)	[a]	–
D. pictus	Early larvae	18.29 (14.93; 21.65)	–	–

TI values < 1.5 indicate low teratogenic potential

TI Teratogenic Index (LC50/EC50)

[a] Only two malformed individuals

Acute toxic effects of RU-UM on early larvae

Mortality was significantly increased in early *X. laevis* larvae (X^2 = 13.16, df = 5, P < 0.05), but Wilcoxon rank sum test between the controls and the highest concentrations did not reach the level of significance (W = 0, P = 0.059). Starting at 36 mg a.i./L, mortality of early *D. pictus* larvae was significantly different from the control ($F_{1,14}$ = 14.45, < 0.01).

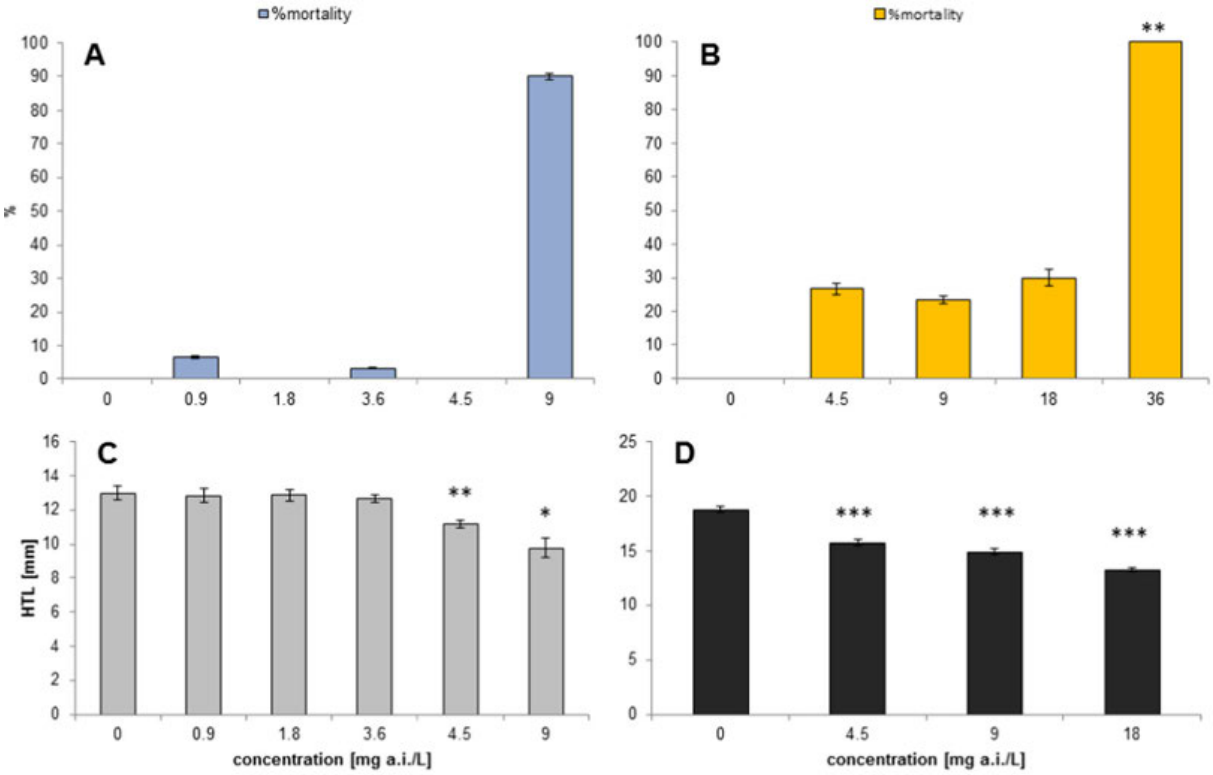


Figure 1 (from Wagner N. *et al.* 2017, ESPR, Vol. 24, No. 2, pp. 1495-1508). Mortality rates in early *X. laevis* (a) and *D. pictus* larvae (b) and growth inhibition due to exposure to Roundup® UltraMax in *X. laevis* (c) and *D. pictus* larvae (d). All values are given ±standard error. Asterisks indicate significant differences to the control (*P<0.05, **P<0.01, ***P<0.001). HTL total length, head to tail length.

Like in embryos, *X. laevis* larvae were significantly more sensitive to exposure to RU-UM if compared to *D. pictus* larvae. NOEC for mortality was 4.5 mg a.i./L in *X. laevis* and 18 mg a.i./L for *D. pictus*. Only one *X. laevis* larva with edema was observed in the control and at 4.5 mg a.i./L one axial malformation. Due to missing concentration dependency, these changes were understood as incidental. No malformed *D. pictus* larvae were found in the control or in the concentration groups. The MCIG in both *X. laevis* ($F_{1,148}$ = 16.67, P < 0.001) and *D. pictus* larvae ($F_{1,94}$ = 171.40, P < 0.001) was 4.5 mg

a.i./L. Consequently, NOEC for growth inhibition in *X. laevis* was 3.6 and <4.5 mg a.i./L in *D. pictus* larvae.

Conclusion

Regarding the authors hypotheses (according to hypothesis number):
(1). In general, *X. laevis* embryos and larvae reacted more sensitive to RU-UM exposure than early developmental stages of *D. pictus*. It was suggested, that beside of unknown properties the different morphology of their filter apparatus and the higher volume of water pumped through the buccopharynx of *X. laevis* lead to higher exposure of the later species to the compound.
(2). RU-UM induced similar lethal effects in *D. pictus* larvae as it does in the teleost standard test organism used in pesticide approval, the rainbow trout (*Oncorhynchus mykiss*), whereas embryos of both species are more tolerant concerning acute lethal effects and, conversely, *X. laevis* larvae about two times more sensitive. Based on calculated surface water concentrations of the a.i., there is, however, an apparent field safety, but buffer strips have to be urgently regarded to any aquatic amphibian habitat.
(3). In both species, early larvae always reacted significantly more sensitive than embryos. Increased metabolism demonstrated by increased growth was understood as the source.
(4). Exposure to RU-UM increased malformation rates in embryos of both species in a concentration-dependent manner, but not at environmentally relevant concentrations. However, the apparent field safety data, based on calculated surface water concentrations of the a.i., should be validated with realistic field data.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested (Roundup® UltraMax, 450 g a.i./L; ether amine ethoxylate as added surfactant) is not the representative formulation for the glyphosate EU renewal. Therefore the article is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

As the tested formulation Roundup® UltraMax does not contain substances forbidden within the EU, the RMS considers the results based on this formulation to be relevant for the risk assessment.

The 96 h LC_{50} values for early larvae are 7.04 and 18.29 mg a.i./L for *Xenopus laevis* and *Discoglossus pictus*, respectively.

The RMS considers the study to be reliable.

B.9.1.4.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

This section includes studies that were concluded by the RMS to be less relevant to the data requirement, primarily because products with unknown co-formulants were tested. These results are however regarded as supplementary and will be used in a WoE approach. These studies were summarised in

detail by the applicant following the subsequent steps of the OECD Guidance documents (OECD, 2005; 2006). The studies have been further evaluated by the RMS and assessed for their reliability.

Data point	CA 8.1.4
Report author	Fuentes L. *et al.*
Report year	2014
Report title	Role of sediments in modifying the toxicity of two Roundup formulations to six species of larval anurans
Document source	Environmental toxicology and chemistry (2014), Vol. 33, No. 11, pp. 2616
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The role of sediment in modifying the toxicity of the original formulation of Roundup and Roundup Weather MAX was examined in aqueous laboratory tests. Six species of anurans (*Bufo fowleri, Hyla chrysoscelis, Rana catesbeiana, Rana clamitans, Rana sphenocephala, and Rana pipiens*) were exposed at Gosner stage 25 to 2 herbicide formulations in 96-h, static, nonrenewal experiments in the presence and absence of sediment.
For the original formulation of Roundup the LC_{50} values ranged from 1.80 mg a.e./L to 4.22 mg a.e./L in water-only exposures and from 4.37 mg a.e./L to 8.26 mg a.e/.L in water-sediment exposures. For the formulation Roundup Weather MAX the LC_{50} values ranged from 1.33 mg a.e./L to 3.26 mg a.e./L in water-only exposures and from 2.94 mg a.e./L to 4.56 mg a.e./L in water-sediment exposures. All species tested had lower median lethal concentration values in water-only exposures of both formulations compared with exposures with sediment. Sediment significantly altered the potency slopes in all tests with the exceptions of *H. chrysoscelis* and *R. clamitans* when exposed to the original formulation of Roundup and *H. chrysoscelis* and *R. sphenocephala* when exposed to Roundup Weather MAX.

Materials and methods

Animals
Six species of Gosner stage 25 anuran amphibians were selected for the present study. Water characteristics were maintained within recommended ranges and similar to conditions in which egg masses were found. Animals were considered acceptable for testing if mortality during the holding period did not exceed 10%.

Sediment
Sediment was collected from an uncontaminated reference site. Average sediment characteristics were as follows: pH 6.87, bulk density 75%, organic matter 1.80%, sand 87.27%, silt 6.30%, clay 6.43%, and total cation exchange capacity 2.7 meq/100 g.

Experimental design
Tests were 96-h, static, nonrenewal exposures. A stock solution of each formulation was prepared with at least 10 nominal concentrations with 4 replicates per concentration. Ten tadpoles were exposed in each replicate, and mortality was recorded at 24 h, 48 h, 72 h, and 96 h. An untreated control was used to represent unexposed conditions. Mortality was indicated by failure to respond to gentle prodding stimuli.

Sample verification
All samples were derivatized and then analyzed for glyphosate based on a standard curve using a Dionex UltiMate-3000 high-performance liquid chromatography system with a variable wavelength detector and auto sampler.

Data analyses
Data were analyzed using SAS1 9.1. Probit regression was used to determine no-observed-effect concentrations (NOECs), lowest-observed-effect concentrations (LOECs), lethal concentration (LCx), and 95% confidence intervals. The USEPA MS-DOS application for trimmed Spearman-Karber analysis was used to obtain median lethal concentration (LC50) values and 95% confidence intervals for nonparametric data, and nonparametric rank converted analysis of variance with Dunnett's test was used to determine NOEC and LOEC values in these cases.

Results

Quality control measures
Control mortality did not exceed 7.5%. Water conductivity averaged 156.0 mS/cm^2 (± 8.3) (range, 144.1-179.0). The mean dissolved oxygen was 8.1 ± 0.9 mg/L (range, 5.2-9.5 mg/L). The initial pH of site water averaged 7.5 ± 0.2 (range, 6.7-7.7). Hardness and alkalinity averaged 35± 4.3 mg of calcium carbonate ($CaCO_3$)/L (range, 26- 44 $CaCO_3$/L) and 41 ± 3.4 mg $CaCO_3$/L (range, 34-50 $CaCO_3$/L), respectively. Temperature was 20 ± 1 °C (68 °F) throughout holding and testing.

96-h LC_{50}s
In 96-h aqueous toxicity tests, LC_{50} values were measured at lower concentrations in all water-only exposures of the original formulation of Roundup and Roundup Weather MAX compared with those exposures with sediment. For the original formulation of Roundup the LC_{50} values ranged from 1.80 mg a.e./L to 4.22 mg a.e./L in water-only exposures and from 4.37 mg a.e./L to 8.26 mg a.e/.L in water-sediment exposures. For the formulation Roundup Weather MAX the LC_{50} values ranged from 1.33 mg a.e./L to 3.26 mg a.e./L in water-only exposures and from 2.94 mg a.e./L to 4.56 mg a.e./L in water-sediment exposures.

Table 1 (from Fuentes L. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2616). Responses of 6 larval anuran species to the original formulation of Roundup in 96-h aqueous and water-sediment exposures.

Species	96-h LC50[a]	NOEC[a]	LOEC[a]	Slope[b]	Threshold[c]	Margin of safety[d]
Aqueous exposures (mg a.e./L glyphosate)						
Rana pipiens	1.80 (1.73–1.88)	1.29 (1.16–1.39)	1.32 (1.19–1.41)	92.5	1.3	—
Rana sphenocephala	2.05 (1.90–2.20)	1.52	1.81	47.9	1.7	—
Hyla chrysoscelis	2.50 (2.38–2.63)	1.74	2.10	44.7	1.9	—
Rana catesbeiana	2.77 (2.66–2.89)	2.02	2.52	66.9	2.3	—
Bufo fowleri	4.21 (4.08–4.33)	3.40	3.95	47.2	3.7	—
Rana clamitans	4.22 (4.02–4.42)	3.27	3.68	24.3	3.5	—
Water–sediment exposures (mg a.e./L glyphosate)						
Hyla chrysoscelis	4.37 (4.17–4.62)	2.76 (2.43–3.00)	2.85 (2.55–3.09)	29.4	2.8	1.0
Rana pipiens	4.83 (4.83–5.16)	2.96	3.86	22.6	3.4	1.1
Rana sphenocephala	5.13 (4.87–5.41)	3.53	4.55	21.3	4.0	1.3
Bufo fowleri	5.84 (5.59–6.10)	4.41	5.20	31.3	4.8	1.6
Rana catesbeiana	6.09 (5.83–6.40)	4.84 (4.35–5.17)	4.90 (4.42–5.21)	14.8	4.9	1.8
Rana clamitans	8.26 (8.04–8.49)	5.34	7.03	21.7	6.2	2.0

[a]Median lethal concentration (LC50), no-observed-effect concentration (NOEC), and lowest-observed-effect concentration (LOEC) with 95% confidence intervals provided if available through probit analysis.
[b]Values expressed as percentage mortality per milligram of acid equivalents per liter (mg a.e./L).
[c]Calculated by averaging NOEC and LOEC.
[d]Based on predicted environmental concentrations for the maximum label application rate assuming a direct overspray and a water depth of 15.2 cm (6 in).

Table 2 (from Fuentes L. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2616). Responses of 6 larval anuran species to Roundup Weather MAX in 96-h aqueous and water-sediment exposures.

Species	96-h LC50[a]	NOEC[a]	LOEC[a]	Slope[b]	Threshold[c]	Margin of safety[d]
Aqueous exposures ((mg a.e./L glyphosate)						
Rana sphenocephala	1.33 (1.22–1.45)	0.68	0.98	54.7	0.8	—
Bufo fowleri	1.96 (1.89–2.04)	1.54 (1.40–1.64)	1.56 (1.42–1.66)	84.2	1.6	—
Rana catesbeiana	1.97 (1.89–2.06)	1.33 (1.16–1.46)	1.37 (1.20–1.49)	63.0	1.4	—
Rana pipiens	2.27 (2.18–2.36)	1.65 (1.48–1.77)	1.68 (1.52–1.80)	65.5	1.7	—
Rana clamitans	2.77 (2.67–2.87)	1.91	2.37	62.2	2.1	—
Hyla chrysoscelis	3.26 (3.14–3.38)	2.48	2.87	49.3	2.7	—
Water–sediment exposures (mg a.e./L glyphosate)						
Rana pipiens	2.94 (2.82–3.07)	2.20	2.60	34.5	2.4	0.8
Rana catesbeiana	3.08 (2.95–3.22)	2.75	3.00	34.9	2.9	1.0
Bufo fowleri	3.54 (3.42–3.67)	2.73 (2.54–2.88)	2.78 (2.59–2.92)	36.5	2.8	1.0
Rana sphenocephala	3.57 (3.46–3.69)	3.25	3.95	44.2	3.6	1.2
Hyla chrysoscelis	3.75 (3.61–3.88)	2.66	2.80	46.2	2.7	1.0
Rana clamitans	4.56 (4.29–4.84)	2.77	3.98	24.1	3.4	1.0

[a]Median lethal concentration (LC50), no-observed-effect concentration (NOEC), and lowest-observed-effect concentration (LOEC) with 95% confidence intervals provided if available through probit analysis.
[b]Values expressed as percentage mortality per milligram of acid equivalents per liter (mg a.e./L).
[c]Calculated by averaging NOEC and LOEC.
[d]Based on predicted environmental concentrations for the maximum label application rate assuming a direct overspray and a water depth of 15.2 cm (6 in).

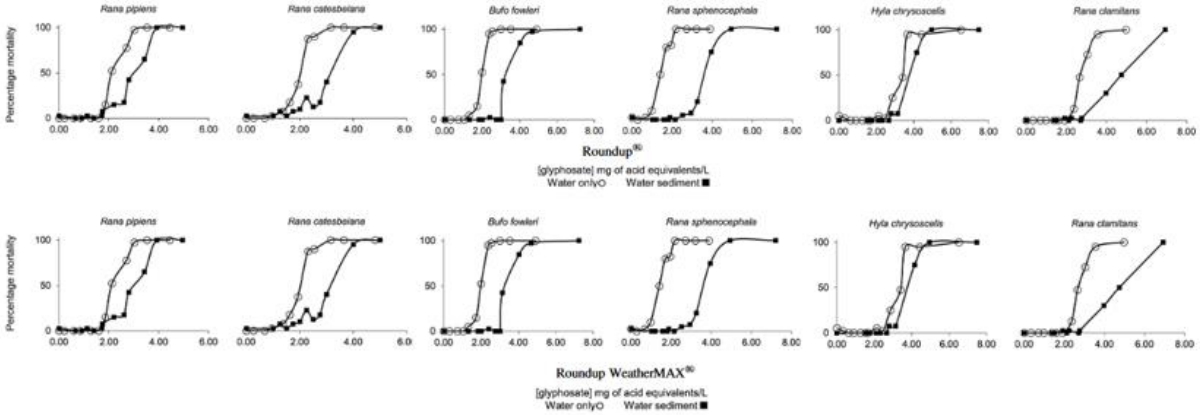


Figure 1 (from Fuentes L. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2616). Concentration-response curves of 6 species of larval anurans to 96-h water-only and water-sediment exposures of the original formulation of Roundup (top) and Roundup Weather Max (bottom).

Acute tests with 6 species of anurans (*Bufo fowleri*, *Hyla chrysoscelis*, *Rana catesbeiana*, *Rana clamitans*, *Rana sphenocephala, and Rana pipiens*) revealed for the original formulation Roundup LC_{50} values between 1.80 mg a.e./L and 4.22 mg a.e./L in water-only exposures and between 4.37 mg a.e./L and 8.26 mg a.e/.L in water-sediment exposures. Test with the formulation Roundup WeatherMAX resulted in LC_{50} values between 1.33 mg a.e./L and 3.26 mg a.e./L in water-only exposures and between 2.94 mg a.e./L and 4.56 mg a.e./L in water-sediment exposures.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: No specific endpoints were presented that could be used in an EU ecotoxicological regulatory risk assessment / glyphosate EU renewal. In addition, none of the tested formulation is the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). Thus the data on the formulations are not relevant for the glyphosate EU renewal.

Further points of clarification:
The original Roundup formulation contains POEA surfactant which is not permitted in formulated herbicidal products in the EU. The influence of POEA on the observed findings cannot be excluded. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The results from the test with the original Roundup formulation are not considered relevant for the assessment of the representative product since this formulation included surfactants similar to substances that are not allowed in the EU (Regulation (EU) 2016/1313 and/or Regulation (EU) 2021/383.

However, Roundup Weather MAX contains an unknown surfactant and thus the results concerning this are seen as less relevant but supplementary and will be used in a WoE approach.

Water-only exposure of 6 larval anuran species to Roundup Weather MAX resulted in 96 h LC_{50} values ranging from 1.33 - 3.26 mg a.e./L. Note that these results are presented in Vol. 1 under the reference Fuentes *et al.* 2011, as it is the same data.

The RMS considers this study to be reliable.

Data point	CA 8.1.4
Report author	Lenkowski J. R. *et al.*
Report year	2010
Report title	Low concentrations of atrazine, glyphosate, 2,4-dichloro-phenoxyacetic acid, and triadimefon exposures have diverse effects on *Xenopus laevis* organ morphogenesis
Document source	Journal of environmental sciences (2010), Vol. 22, No. 9, pp. 1305
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

X. laevis tadpoles were exposed to Roundup concentrations of 0.25, 0.5, 1 and 5 mg product/L for 48 hr with static renewal at 24 hr. Glyphosate (RoundUp) exposures caused no mortality but a significant increase in intestinal malformations only at 5 mg a.s./L.

Materials and methods

X. laevis tadpoles were cultured in 0.1 × Marc's Modified Ringer's solution (MMR; 0.01 mol/L NaCl, 0.2 mmol/L KCl, 0.1 mmol/L $MgCl_2$, 0.2 mmol/L $CaCl_2$, 0.5 mmol/L HEPES, pH 7.5), euthanized with MS-222, and fixed 1 hr at room temperature in MEMFA (0.1 mol/L MOPS, pH 7.5, 2 mmol/L EGTA, 1 mmol/L MgSO4, 3.7% formaldehyde). Working dilutions were made in 0.1X MMR (Table 1).

X. laevis tadpoles were exposed to Roundup concentrations of 0.25, 0.5, 1 and 5 mg a.s./L for 48 hr with static renewal at 24 hr. Exposures began at NF stage 41, were performed in triplicates, and maintained at 18°C. Tadpole density per exposure was 13-18 tadpoles in 10 mL of MMR.

Table 1 (from Lenkowski J. R. *et al.* 2010, J Environ Sci, Vol. 22, No. 9, pp. 1305). Pesticides, stocks, and working concentrations used in experiments.

Pesticide	Vehicle	Stock (mg/mL)	Working concentration (mg/L)
Glyphosate (RoundUp®)	0.1X MMR	180	0.25, 0.5, 1, 5

MMR: Marc's Modified Ringer's solution.

Results

Glyphosate (RoundUp) exposures caused no mortality but a significant increase in intestinal malformations only at 5 mg/L active ingredient (Fig. 1).

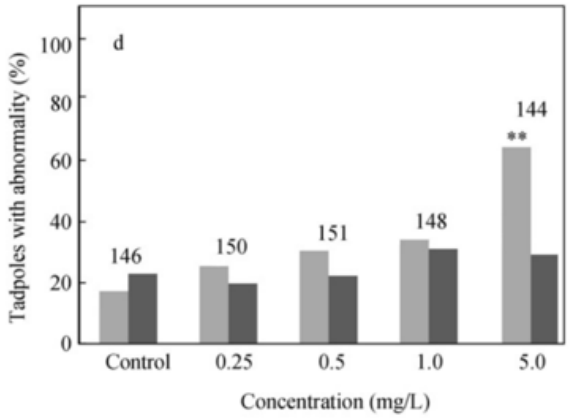


Figure 1 (from Lenkowski J. R. *et al.* 2010, J Environ Sci, Vol. 22, No. 9, pp. 1305)**:** Incidence of intestine malformations ■ and edemas ■ in tadpoles exposed to glyphosate (d) starting at NF stage 41 for 48 hr. Sample size above bars; ** $p < 0.005$.

Glyphosate (RoundUp) exposures caused no mortality but a significant increase in intestinal malformations only at 5 mg/L active ingredient.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Toxicity of glyphosate and other chemistry to amphibians to assess malformations, up to 5 mg/L. Static renewal at 24 hr in 48 hr study. Conducted in the US. The described endpoints are not relatable to an EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Further points of clarification:
The test substance purity and identity cannot be confirmed from the information presented in the paper. The original Roundup formulation contains POEA surfactant which is not permitted in formulated herbicidal products in the EU. The surfactant system in the formulated product used in this study, was not confirmed by the authors. Therefore, the findings of this paper should be treated with a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

A significant increase (by a factor of 3 compared to the control) in intestinal malformations was observed in *X. laevis* tadpoles after 48 h exposure to Roundup (5 mg a.i./L). NOEC = 1 mg a.i./L. Although not a standard endpoint for risk assessment, this observation may have an impact on the overall fitness of individuals and on natural populations.

The RMS considers this study as reliable with restrictions, as nominal test concentrations were not analytically confirmed.

Data point	CA 8.1.4
Report author	Williams B. K. *et al.*
Report year	2010
Report title	Larval responses of three midwestern anurans to chronic, low-dose exposures of four herbicides
Document source	Archives of environmental contamination and toxicology (2010), Vol. 58, No. 3, pp. 819-827
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

dossier (KCA 9) by the applicant;
See also RMS analysis in RMS box

This study aimed to expose three common species of tadpoles to conservative levels of glyphosate end-use herbicide formulations throughout the larval period to test for survival differences or life-history trait alterations. Exposure to the glyphosate product Roundup WeatherMax® at 572 ppb glyphosate acid equivalents (a.e.) resulted in 80% mortality of western chorus frog tadpoles, likely as a result of a unique surfactant formulation. Exposure to WeatherMax® or Roundup Original Max® at 572 ppb a.e. also lengthened the larval period for American toads.

Materials and Methods

Animal collection
Multiple partial egg strings (*B. americanus*) or masses (*P. triseriata*) were collected from two wetlands in Union Ridge Conservation Area (Sullivan and Adair counties in Missouri, USA) on April 16, 2005. *Hyla versicolor* were collected on June 14, 2005 as four amplexed pairs from a pond in the Thomas S. Baskett Wildlife Research and Education Area (Boone County, MO, USA), and allowed to oviposit in the laboratory. Both collection sites are protected areas with low impact from agricultural activities. All eggs were maintained at 25 °C through hatching until experiments began. At Stage 25, tadpoles were thoroughly mixed within species in order to homogenize variation among clutches and then randomly assigned to an individual glass jar. Any unused tadpoles were returned to the original collection site. Following the natural breeding phenology of the species, experiments with *B. americanus* and *P. triseriata* began on April 25, 2005 and the *H. versicolor* experiment began on June 23, 2005.

Experimental Design
Tadpoles were exposed to two commercially available herbicide products, two formulations containing glyphosate potassium salts. End-use herbicide formulations were used because these are the forms to which organisms in the field would be exposed. Tadpoles were raised individually in 1-L glass jars and were randomly assigned to a single herbicide treatment, glyphosate (0.6 ppb or 700 ppb a.i. for each of the two formulations), with 10 replicates per treatment. Treatment concentrations for each herbicide were drawn directly from stream water quality monitoring averages and from drinking water standards (Table 1). Although source data for treatment levels were listed in active ingredient concentrations, glyphosate concentrations are usually expressed in glyphosate acid equivalents (a.e.) to facilitate comparison between formulations with different glyphosate salts. For both glyphosate formulations of interest, active ingredient concentrations are multiplied by 0.81 to yield acid equivalent concentrations; hence, treatment concentrations were 0.5 ppb and 572 ppb a.e. Hereafter all glyphosate concentrations will be reported in acid equivalents. A stock solution for each chemical treatment was created by mixing end-use herbicide products with reagent-quality distilled water. All stock solutions were stored at 3 °C in a foil-covered glass jar. At each renewal, 0.1 mL of stock solution was pipetted into each jar to yield the desired final concentration of active ingredient. Glyphosate was added as Roundup Original Max® (48.7% glyphosate potassium salt) or Roundup WeatherMax® (48.8% glyphosate potassium salt; Monsanto, St. Louis, MO, USA). Control jars received a sham treatment of 0.1 mL of deionized water. Test water for all treatments (control and herbicide) was ultraviolet (UV)-disinfected, carbon-filtered water (conductivity: ~ 688 µS/cm; pH: ~ 7.8; total alkalinity: ~ 270; total hardness: ~ 232). Tests were conducted at 25 °C with a light cycle of 16L:8D. Because of slight vertical gradients in temperature, jars were arranged in blocks according to shelf height. Water in jars was changed and chemical treatments were renewed every 3 days. Tadpoles were fed a 50/50 mixture of ground Tetramin® fish flakes and ground commercial rabbit chow (*B. americanus* and *P. triseriata*) or 100% ground fish flakes (H. versicolor) after each water change, beginning at 4.5 mg/tadpole and increasing periodically as animals grew.

Table 1 (from Williams B. K. *et al.* 2010, AECT, Vol. 58, No. 3, pp. 819-827). Herbicide treatments and stream water quality source data.

Active ingredient	Treatment level (ppb a.i.)	Source data type	Treatment calculation	Ref.
Glyphosate	0.6	Pre-, post-emergence, and harvest season samples	Average over 51 sites when detected	Scribner et al. (2003)
	700	Drinking water standard (MCL)	NA	USEPA (2006)

Response variables and statistical analysis

The following was measured: survival, mass at metamorphosis, and length of the larval period to assay the response of tadpoles to experimental treatments. Survival was calculated by dividing the number of surviving tadpoles and metamorphs by the number of tadpoles initially added. All *B. americanus* and *P. triseriata* survivors metamorphosed, whereas 26% of initial *H. versicolor* tadpoles had not metamorphosed by the termination of the experiment. Metamorphosis was defined as emergence of at least one forelimb (stage 42). Each metamorph was held in an individual plastic container until tail resorption was complete (mean = 3 days) and then blotted dry and weighed to the nearest 0.1 mg. Survival in each species was analyzed with logistic regression to detect differences among treatments. Mass at metamorphosis and time to metamorphosis were evaluated using univariate analyses of variance with $\alpha = 0.05$. In the case of a significant treatment effect, treatment means were compared using a Tukey-Kramer adjustment for multiple comparisons. All analyses were conducted using SAS (version 9.1; SAS Institute, Cary, NC, USA). Power analyses were performed a posteriori for mass at metamorphosis and time to metamorphosis. Block effects were not significant in any test, with the exception of larval period length for *B. americanus*. In this case, block was included as a treatment in the analysis of treatment effects.

Results

Exposure of *P. triseriata* tadpoles to 572 ppb a.e. glyphosate in the form of Roundup WeatherMax resulted in high mortality, with only 20% of exposed animals surviving through metamorphosis (Fig. 1). This value was significantly different from the control treatment ($p = 0.0131$). Survival of *B. americanus* and *H. versicolor* tadpoles was unaffected by herbicide exposure. Tadpoles metamorphosed 14% later when exposed to 572 ppb a.e. glyphosate as Roundup WeatherMax (27.8 ± 0.4 days) or 8% later with Roundup Original Max® (26.2 ± 0.6 days), when compared to control animals (24.3 ± 0.3 days). Herbicide treatment had a marginally significant effect on time to metamorphosis in *P. triseriata* ($F_{8, 54} = 2.11$, $p = 0.0504$; Fig. 2). The mean larval period of *P. triseriata* tadpoles exposed to 572 ppb a.e. glyphosate as Roundup WeatherMax was 13% longer (32.0 ± 1.0 days) than that of control animals (28.3 ± 0.7 days). However, this result should be viewed with caution, considering that high mortality in the WeatherMax treatment left only two animals for the larval period length calculation. Larval periods for *H. versicolor* tadpoles were not affected by herbicide treatment ($p = 0.3711$). Power analyses indicated that power to detect significant differences among larval period lengths was high for *B. americanus* and *P. triseriata* (power = 0.981 and 0.998, respectively) and moderate for *H. versicolor* (power = 0.536). Mass at metamorphosis did not differ with herbicide treatment for any of the three species tested (*B. americanus*, $p = 0.8530$; *P. triseriata*, $p = 0.6863$; *H. versicolor*, $p = 0.6100$), although power was relatively low (power = 0.152, 0.202, and 0.345, respectively).

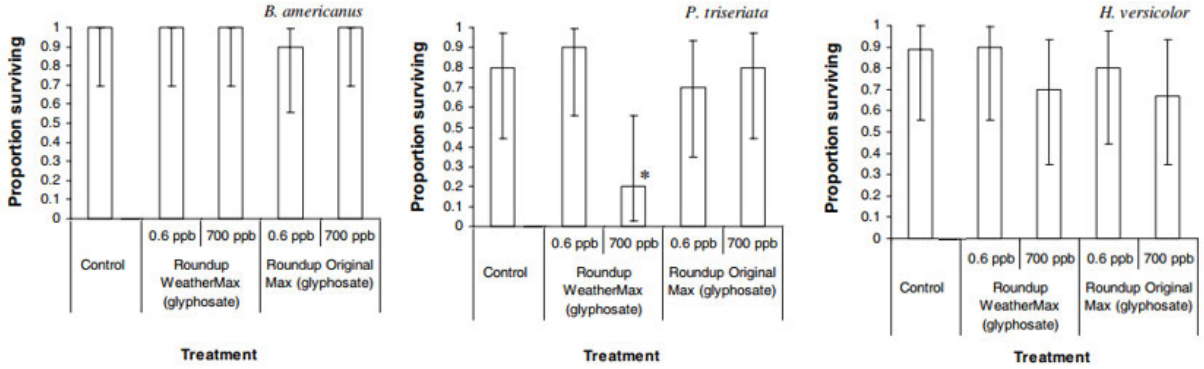


Figure 1 (from Williams B. K. *et al.* 2010, AECT, Vol. 58, No. 3, pp. 819-827). Survival of three species of anuran larvae exposed to common herbicide formulations. One animal each from the *H. versicolor* control and 700-ppb glyphosate treatments was injured during the experiment and excluded from the analysis. For all other treatments, n = 10. Error bars represent 95% confidence intervals. An asterisk indicates survival significantly different ($p < 0.05$) from the control treatment.

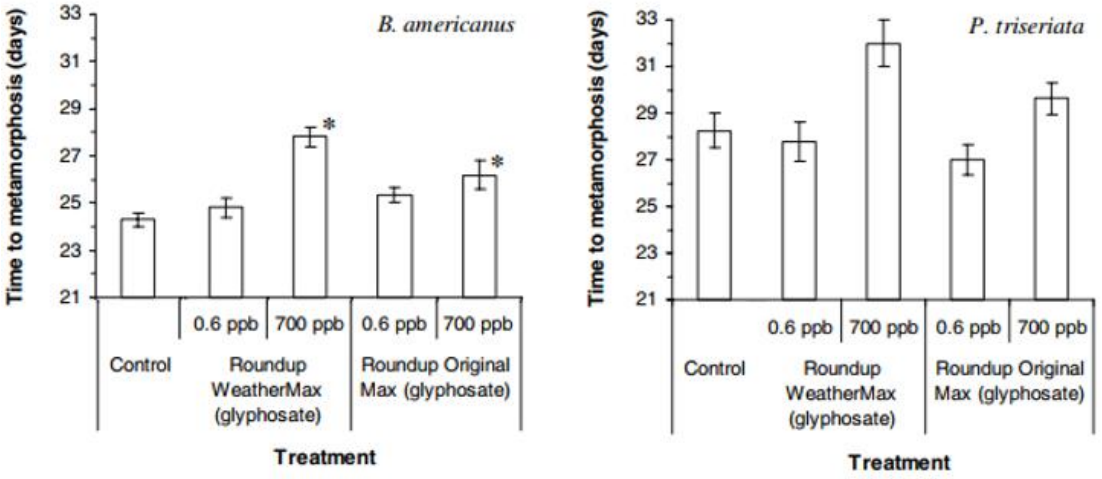


Figure 2 (from Williams B. K. *et al.* 2010, AECT, Vol. 58, No. 3, pp. 819-827). Time to metamorphosis for *B. americanus* and *P. triseriata* tadpoles exposed to common herbicide formulations. Error bars are ±1 SE and an asterisk indicates larval periods significantly different ($p < 0.05$) from controls.

These results indicate that herbicide products commonly detected in stream water have the potential to cause mortality and alter life-history traits in larval anurans, even at levels as low as Environmental Protection Agency (EPA) drinking water standards. At a level of glyphosate intended to be protective of human health (700 ppb a.i., 572 ppb a.e.), exposure to the glyphosate formulation Roundup WeatherMax resulted in 80% mortality of chorus frog tadpoles. Comparable mortality was not seen in the Roundup Original Max treatment, although both formulations contained the same level of glyphosate acid (572 ppb acid equivalents, a.e.). Mortality in the current study appears to be associated with surfactant/adjuvant components present in Roundup WeatherMax but absent from the Roundup Original Max formulation. Treatment concentrations in this study were intended to elicit sublethal effects; therefore, the chorus frog mortality was unexpected. The higher of the two glyphosate treatment concentrations (572 ppb a.e.) is below the amphibian chronic toxicity reference value (TRV) of 740 ppb a.e. calculated for glyphosate. Chorus frog mortality might also have been influenced by the pH of the test water. The pH of the test water in the current study (7.8) might have increased the gill accumulation of surfactant/ adjuvant components from the WeatherMax formulation, resulting in chorus frog mortality at 572 ppb a.e. In addition to chorus frog mortality, exposure to Roundup WeatherMax and Roundup Original Max at 572 ppb a.e. lengthened the larval period for American toad tadpoles. Animals in these two treatments metamorphosed an average of 4 days (14%) and 2 days (8%) later, respectively, than control animals. These differences, although statistically significant, are of unknown biological importance. In the study, the lower exposure levels for each herbicide were based on concentrations

detected in streams across the Midwest. Because these concentrations were averaged both over time and over many sampling sites, the final treatment levels are extremely conservative estimates of non-target aquatic organism exposure to herbicide products.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Eggs collected from wetlands. A study which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). None of the tested formulations is the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning regarding the difficulty of extrapolating the results to the EU level, since this is a laboratory study.

Further, there is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The results of the study indicate that exposure to both WeatherMax and Roundup Original Max at 572 ppb a.e. lengthened the larval period of *Bufo americanus*, the latter formulation also resulting in 80% mortality of *Pseudacris triseriata* at 572 ppb a.e. Thus, NOEC for survival of *P. triseriata* upon exposure to Roundup WeatherMax and NOEC for time to metamorphosis of *B. americanus* (upon exposure to both Roundup WeatherMax and Roundup Original Max) are determined to be 0.49 ppb a.e.

The RMS considers this study to be relevant and reliable with restrictions, as no analytical confirmation of test concentrations was reported in the study.

Data point	CA 9
Report author	Agostini M. G. *et al.*
Report year	2020
Report title	Pesticides in the real world: The consequences of GMO-based intensive agriculture on native amphibians
Document source	Biological Conservation (2020), Vol. 241, Article ID 108355
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

Pesticide use has been suggested as one of the major drivers of the global amphibian decline. Using in-situ enclosures, the authors evaluated the effects (survival and mobility) of common pesticides applied by farmers (cypermethrin, chlorpyrifos, endosulfan, glyphosate, and 2,4-dichlorophenoxyacetic acid) on tadpoles. Four common amphibian species from South America, across 91 ponds located in the Pampas of central Argentina were studied. Survival decreased in 13 out of 20 pesticides applications concomitantly with detection of pesticides in water ponds. 48 h after applications, mixtures containing endosulfan or chlorpyrifos reduced tadpole survival to < 1% while the cypermethrin mixtures reduced survival to 10%. There was mobility impairment in all combination of pesticides, including glyphosate. The ecological context involved in this study represents the common exposure scenarios related to GMO-based agriculture practices in South America, with relevance at regional levels. The authors emphasize that multifaceted approaches developed to understand the role of pesticides in the amphibian decline need a conservation perspective.

Materials and methods

Test material:	Pesticides active substances were named, but identities not confirmed in article. Pesticides used were: cypermethrin, chlorpyrifos, endosulfan, glyphosate, and 2,4-dichlorophenoxyacetic acid
Exposure concentrations:	Not stated – all pesticide applications were decided and conducted by farmers depending on the demands of the crops.
Test organism:	
Species:	Tadpoles from the following family / Species: Hylidae - *B. pulchellus* Leptodactylidae - *L. latrans* Bufonidae - *R. fernandezae and R. arenarum*
Size:	Tadpoles
Age:	Post hatch tadpoles Gosner stage 29 - 42
Source:	Naturally occurring populations in wild pond sites
Test design:	
Test duration:	48-hour post application by farmer to farmland adjacent to pond sites.
Replication:	Five replicates per pond.
Tadpoles per enclosure:	20 fish per replicate
Test volume (L):	Not stated
Loading:	Unknown
Feeding:	None
Acclimation:	7 days prior to farmer application to adjacent field.
Observations:	At 48 hours before application and then at 24 (T1) and 48 (T2) hr post application. Tadpoles removed from enclosure using fine mesh net and placed into plastic box containing pond water and observed for 5 mins on each observation occasion.
Environmental conditions:	Temperature, dissolved oxygen –DO- conductivity and pH

Abiotic parameters of the ponds assessed (data are given as mean values and standard errors).
T: temperature. **DO**: dissolved oxygen. **C**: conductivity.

Pond identity	T (°C)[a]	DO (mg/L)[b]	C (mS/cm)[c]	pH[d]
Ponds adjents to crops (n = 71)	20.70 ± 0.96	9.23 ± 0.95	0.21 ± 0.06	7.05 ± 0.64
Reference ponds (n = 20)	20.80 ± 1.05	9.05 ± 0.80	0.19 ± 0.05	7.19 ± 0.78

[a] $F = 0.01$, $df = 1–51$, $p = 0.91$.
[b] $F = 0.56$, $df = 1–51$, $p = 0.46$.
[c] $F = 1.24$, $df = 1–51$, $p = 0.27$.
[d] $F = 0.06$, $df = 1–51$, $p = 0.79$.
(from Agostini M. G. *et al.* 2020, Biol. Conserv, Vol. 241)

Results

Following application – pesticides detected in 63 out of 71 ponds adjacent to the crop. The results including tadpole survival, mortality and the range of pesticide detected (µg/L) are presented in the following table.

Table 1 (from Agostini M. G. *et al.* 2020, Biol. Conserv, Vol. 241). Percentages of survival and impairment of mobility across time in tadpoles from ponds receiving pesticides and ranges of pesticides concentration detected in water samples. AP: number of pesticide application assessed. Area: (A: South of Rolling Pampas, B: Central of Rolling Pampas, C: North of Rolling Pampas). CP: chlorpyrifos. ENDO: endosulfan. CY: cypermethrin. GLY: glyphosate, 2,4-D: 2,4-dichlorophenoxyacetic acid. Bp: *Boana pulchellus*, Ll: *Leptodactylus latrans*, Rf: *Rhinella fernandezae*, Ra: *Rhinella arenarum*. T0: 24 h before application. T1: 24 h after application. T2: 48 h after application.

AP + Area	Pesticides applied	Pond ID	Species	Tadpole survival			Tadpole mobility		Range of pesticide detected µg/L
				T0	T1	T2	T1	T2	
1A	CY-GLY	C1–3	*Bp*	100	38.6 (± 8.7)*	18 (± 11.7)φ	56 (± 15.5)*	87.1 (± 12.8)φ	CY 195.3–365.35 - GLY 153.5–231.2
3A	CY-GLY	C7–8	*Ll*	100	27.5 (± 10.6)*	1 (± 2.1)φ	85.4 (± 2.0)*	–	CY 102.3–214.8 - GLY 67.3–137.2 **END 3.9**
4A	CY-GLY	C9–10	*Rf*	99	30.1 (± 7.3)*	2.5 (± 2.6)φ	85 (± 13.8)*	–	CY 149.5–184.5 - GLY 18.2–35.7
5B	CY-GLY	C11–14	*Ra*	100	29.5 (± 5.5)*	1 (± 2)φ	71.1 (± 0.9)*	–	CY 124.0–286.0 - GLY 89.2–320.7
6B	CY-GLY	C16–19	*Bp*	100	41.5 (± 9.7)*	3.5 (± 3.2)φ	69.2 (± 5.5)*	–	CY 239.3–354.9 - GLY 99.2–188.2
7C	CY-GLY	C20–23	*Bp*	100	20.2 (± 1.7)*	9.2 (± 5.5)φ	85.1 (± 13.2)*		CY 114.5–330.9 - GLY 73.4–156.8 **CP 1.5**
8C	CY-GLY	C25–28	*Rf*	100	29.6 (± 8.3)*	6 (± 3.8)φ	78.6 (± 9.2)*	–	CY 231–413.9 - GLY 105.4–211.8
9C	CY-GLY	C29–31	*Bp*	100	37.2 (± 11.2)*	35.5 (± 12.9)	59.3 (± 11.8)*	75.3 (± 13.7)φ	CY 122.9–215.6 - GLY 121.2–196.9
10A	CP-GLY	C33–34	*Ll*	99	7.5 (± 4.2)*	1.5 (± 2.4)φ	–	–	CP 245.3–256.6 - GLY 31.2–87.9
11B	CP-GLY	C35–39	*Bp*	100	1.6 (± 2.2)*	0	–	–	CP 176.9–240.1 - GLY 104.2–155.3
12B	CP-GLY	C40–42	*Bp*	99	5.6 (± 4.5)*	5 (± 4.6)	–	–	CP 211.3–230.6 - GLY 98.8–105.7
13A	END	C44–45	*Rf*	99.9	4 (± 4.5)*	0.5 (± 1.6)	–	–	END 242.9–327.5
14A	CY-GLY-END	C46	*Ll*	100	2 (± 4.4)*	0	–	–	CY 45.6 - GLY < 0.5 - END 230.3
15B	GLY-2,4-D	C47–51	*Rf*	100	79.6 (± 13.3)*	76.6 (± 11.5)	76.8 (± 6.1)*	77.2 (± 4.7)	GLY 178.5–330.3 - 2,4-D 70.4–180.1 **END 1.4–1.6**
16B	GLY-2,4-D	C52–57	*Rf*	100	99.3 (± 2.1)	98.8 (± 2.8)	61.2 (± 6.1)*	71.5 (± 5.7)φ	GLY 56.8–83.4 - 2,4-D 78.9–101.4
17C	GLY-2,4-D	C58–61	*Bp*	99	98.6 (± 2.9)	96.9 (± 4.5)	79.3 (± 3.3)*	93.1 (± 4.8)φ	GLY 173.5–190.3 - 2,4-D 132.4–209.6
18A	GLY	C62–64	*Bp*	99	99.4 (± 1.5)	98.9 (± 2.1)	48.8 (± 11.4)*	65.1 (± 6.4)φ	GLY 110.5–179.3
19A	GLY	C66–67	*Bp*	100	100	99.5 (± 1.5)	67 (± 5.3)*	79.8 (± 3.9)φ	GLY 54.5–92.5 **END 0.9**
20C	GLY	C68–71	*Bp*	100	99.2 (± 1.8)	98.7 (± 2.2)	74.1 (± 4.3)*	94.1 (± 4.1)φ	GLY 214.5–315.5

– Effect not evaluated because the low number of tadpoles surviving.
Bold concentrations are indicating pesticides not applied but detected in water samples.
* Significant effects compared with T0 (Tukey's post hoc analysis: $p < 0.05$).
φ Significant effects compared with T1 (Tukey's post hoc analysis: $p < 0.05$).

Tadpole mobility

Results (Table 1) showed that the mobility of surviving tadpoles was negatively affected by all combination of pesticides. The applications involving cypermethrin + glyphosate mixture (1A, 3-4A, 5-6B and 7-9C) caused a significant ($p < 0.05$) decrease of mobility in tadpoles exposed to 73.7% (± 11.6) at T1 and 95.3% (± 9.2) at T2.

The authors also found significant effects ($p < 0.05$) on mobility in tadpoles exposed to the six applications involving glyphosate and glyphosate + 2,4-D mixture (15-16B, 17C, 18-19A and 20C). After application of glyphosate + 2,4-D mixture, 84.5% (± 11.6) of surviving tadpoles were negatively affected, while 79.4% (± 14.5) tadpoles were affected after glyphosate applications. The effects on

mobility were significantly (Tukey's post hoc analysis: $p < 0.05$) higher at T2 than those observed at T1 in 7 of the 8 applications where we were able to conduct the analyses. The low number of surviving tadpoles exposed to endosulfan (13A), chlorpyrifos + glyphosate mixtures (10A, 11-12B) and cypermethrin + glyphosate + endosulfan mixture (14A) did not allow them to conduct the evaluation on mobility endpoint (Table 1). No effects were detected in tadpoles placed in the reference pond enclosures.

Tadpole survival
The authors found that survival significantly decreased ($p < 0.05$) after 13 out of the 20 pesticide applications (Table 1). All pesticide applications involving endosulfan caused a significant decrease ($p < 0.05$) of survival at T1 and T2. After application of endosulfan (13A), only 0.5% (± 1.5) of tadpoles survived at T2, while no tadpoles survived after the application of cypermethrin + glyphosate + endosulfan (14A). All the applications of chlorpyrifos + glyphosate (10A, 11B and 12B) reduced survival to 4% (± 4.4) at T1 and 1.8% (± 3.4) at T2. Except for 2A where pesticides did not reach the pond, all the applications involving cypermethrin + glyphosate mixture (1A, 3-4A, 5-6B and 7-9C) caused a significant decrease ($p < 0.05$) on survival to 31.6% (± 11.2) and 10.5% (± 13.5) at T1 and T2 respectively. Only one application of glyphosate + 2,4-D mixture (15B) caused a significant decrease of tadpole survival ($p < 0.05$) reducing the survival to a 76.6% (± 11.5). None of the applications of glyphosate were associated with a significant decrease in survival ($p > 0.05$). The analyses also showed that survival at T2 was significantly lower (Tukey's post hoc analysis: $p < 0.05$) compared with those obtained at T1 in 8 of the 13 applications (1A, 3-4A, 5-6B and 7-9C) (Table 3). The total average of survival detected in control ponds was 99.95% (± 0.21), 99.91% (± 0.30), 99.8% (± 0.41) at T0, T1 and T2, respectively.

Conclusion

The authors highlighted that the work has triggered alarm about the detrimental impact of pesticides (insecticides and herbicides) on native amphibians inhabiting the shallow ponds of the richest agricultural lands of South America. They documented effects caused by pesticides on tadpoles which can compromise the viability of populations living in agricultural landscapes. The intensive agricultural model based on the GMO technological package currently applied in South America is expected to expand (and intensify) over the coming years. Therefore, it is also expected that native amphibian populations will continue being affected. The authors suggested that conservation priorities should be focused on developing a better policy legislation for pesticide use, including not only the protection of human settlements but also native terrestrial and wetland habitats. Additionally, there is an urgent need to build knowledge on the alternative management practices (e.g. borders management, crop rotation) to the extended and intensive use of pesticides for pest control. Finally, it is necessary to extend the knowledge on the effects of pesticide applications not only for other amphibian species but also for other taxa conforming aquatic communities with the purpose to achieve a more accurate understanding of the conflict involving pesticide use. This will enable to predict and mitigate future pesticides impacts and establish conservation priorities for species, critical habitats, and ecosystems.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). Observations are caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity - this paper looks at the impact of mixtures of pesticides rather than single actives). In addition, it is not clear which glyphosate-based herbicide was exactly tested. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Further points of clarification:

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS notes a mismatch in the species listed for area 20C: the paper says *Ra* in Table 1 but *Bp* in Table 3. We assume that the first one is correct.

The RMS does not agree with the applicant's reasoning and conclusion regarding relevance for the EU assessment.

This paper did not investigate only mixtures, but also individual substances including glyphosate.

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

Differences in factors such as geoclimatic properties and land-uses etc are also not considered as relevant in this context, as the biological endpoints are related to the actual measured glyphosate concentrations in ponds.
Although no significant mortality was observed from glyphosate treatment, tadpole mobility was significantly reduced in test ponds 24 h after spraying. The measured glyphosate concentrations in pond water ranged 54.5-315.5 µg/L. Hence, NOEC for survival was 315.5 µg/L for *Rhinella arenarum* and 179.3 µg/L for *Boana pulchellus*, whereas NOEC for mobility could not be determined for any of the 2 species tested, thus LOEC is 54.5 µg/L for *B. pulchellus* and 214.5 µg/L for *R. arenarum* (i.e., the lowest glyphosate concentrations measured in the test ponds).

The RMS considers this study to be reliable.

Data point	CA 9
Report author	Edge C. *et al.*
Report year	2014
Report title	Variation in amphibian response to two formulations of glyphosate-based herbicides
Document source	Environmental toxicology and chemistry (2014), Vol. 33, No. 11, pp. 2628-32
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The authors exposed larval wood frogs (*Lithobates sylvaticus*) from 4 populations to two glyphosate-based herbicides, Roundup Weed and Grass Control® and Roundup WeatherMax®. The 96-h median lethal concentration values for both formulations varied among the populations (Roundup Weed and Grass Control, 0.59 mg a.e./L to 1.10 mg a.e./L; Roundup WeatherMax, 4.94 mg a.e./L to 8.26 mg a.e./L), demonstrating that toxicity varies among the formulations and that susceptibility may differ among populations.

Materials and Methods

Study animals
Between 5 and 10 wood frog egg masses were collected from a single wetland each in Blanchester (OH, USA), Ottawa (Eastern Ontario, Canada), and the Long-Term Experimental Wetlands Area in Gagetown (New Brunswick, Canada) and from each of 3 wetlands in Sault Ste. Marie (Central Ontario, Canada). Eggs were shipped overnight, kept cool by packing with ice, to the University of New Brunswick in Saint John, Canada. When they arrived in Saint John, egg masses were maintained at 13 °C on a 12:12 light:dark cycle in an environmental chamber until larvae reached Gosner stage 25.

Experimental set-up
Due to different climates at each of the collection sites, egg laying was not synchronous; therefore, 4 separate experiments were conducted chronologically as egg masses were laid. The order of populations was Ohio, Eastern Ontario, New Brunswick, and Central Ontario. Animals from each population were exposed to 9 concentrations, with each concentration replicated 6 times. The formulations Roundup Weed and Grass Control (5.18 g a.e./L present as the isopropylamine salt) and the formulation Roundup WeatherMax (540 g a.e./L present as the potassium salt;) were used.

Duplicate 45-mL water samples were taken from each dilution and immediately frozen for later analysis. For each population, animals from each of the egg masses were pooled, and 10 animals were chosen at random and placed in each container. The containers were then assigned randomly to a location on a laboratory bench. This resulted in 54 experimental units (9 concentrations x 6 replicates) for each formulation and a total of 108 experimental units (54 experimental units for each of 2 different formulations) for each population. To estimate the amount of variation expected among exposures within the same population, the researchers replicated the experiment using the 3 wetlands from the Central Ontario site as replicates for 1 formulation, Roundup Weed and Grass Control.

Due to a shortage of animals, only 4 control replicates and 5 concentrations (0 mg a.e./L, 0.5 mg a.e./L, 1.0 mg a.e./L, 1.5 mg a.e./L, and 2.0 mg a.e./L) were conducted for replicate 3. Exposures were conducted under static conditions without water renewal, and animals were not fed during the exposure.

Based on previously reported LC50 values, the researchers began the exposures with the following concentrations: 0 mg a.e./L, 0.5 mg a.e./L, 1.0 mg a.e./L, 1.5 mg a.e./L, 2.0 mg a.e./L, 2.5 mg a.e./L, 3.0 mg a.e./L, 3.5 mg a.e./L, and 4.0 mg a.e./L. If no mortality was observed for 1 of the herbicide formulations, the concentrations were doubled for the next replicate test population until the researchers were able to estimate an LC50 value for that formulation. Doubling the exposure concentrations occurred for the Roundup WeatherMax exposures only (see Results). Animals were counted, and dead animals removed 24 h, 48 h, and 72 h after the experiment began. The experiment was terminated after 96 h.

Water quality and exposure validation
All exposures were conducted in the same room at a temperature between 20 °C and 22 °C and a water pH of 5.7. The water samples were frozen and shipped overnight on ice to the laboratory services branch of the Ontario Ministry of the Environment in Etobicoke, Ontario, Canada. Quantitative analysis of glyphosate concentration was conducted using liquid chromatography-tandem mass spectrometry following previously described methodology.´

Statistical analyses
We estimated 96-h 10% lethal concentration (LC_{10}) and LC_{50} values for each population using Probit regression. To test for differences in the mortality percentage between populations after 96 h, the researchers used analysis of covariance (ANCOVA), with population as the subject factor, concentration as the covariate, and mortality as the response variable. The mortality percentage was arc sin square root transformed prior to analyses. These analyses were carried out on the linear portion of the response curve between the lowest concentration at which mortality was observed and the concentration at which 100% mortality was observed. The researchers quantitatively compared the difference in LC_{10} and LC_{50} values between populations with their within-population estimate of variability for the Sault Ste. Marie population.

Results

In all populations and formulations, survival in control replicates was greater than or equal to 90%; therefore, observed mortality was not corrected for control mortality. For Roundup Weed and Grass Control, 96-h LC_{50} values ranged from 0.085 mg a.e./L to 1.1 mg a.e./L (Table 1). Among populations, LC_{50} values differed by as much as 1.015 mg a.e./L, and LC_{10} values differed by 0.88 mg a.e./L. Because all animals survived in the control and all animals died at concentrations at and above 2.0 mg a.e./L, only data from the 0.5 mg a.e./L, 1.0 mg a.e./L, and 1.5 mg a.e./L treatments were used in the ANCOVA to test for differences in the slope of the response among populations. Overall, there were differences in mortality among the populations ($p < 0.001$). Mortality increased with exposure concentration, and the slope of the response curve differed among populations ($p < 0.001$). Both the quantitative comparison of LC_{50} and LC_{10} values and the results of the ANCOVA indicate that the toxicity of Roundup Weed and Grass Control to wood frog larvae differed among the populations (Fig. 1).

Table 1 (from Edge C. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2628-32). Lethal concentrations for 10% and 50% (LC_{10} and LC_{50}, respectively) of 4 populations of *Lithobates sylvaticus* tadpoles exposed to 9 concentrations of 2 herbicide formulations[a].

	Roundup Weed and Grass Killer		Roundup WeatherMax	
Population	LC10	LC50	LC10	LC50
Ohio	0.90 (0.84, 0.97)	1.10 (1.046, 1.16)		>4
Eastern Ontario	0.38 (0.31, 0.46)	0.71 (0.63, 0.80)		>8
New Brunswick	0.33 (0.10, 1.079)	0.58 (0.52, 0.66)	1.06 (0.67, 1.69)	4.94 (4.18, 5.83)
Central Ontario replicate 1	0.73 (0.64, 0.83)	0.99 (0.92, 1.06)	5.98 (5.28, 6.77)	8.26 (7.71, 8.83)
Central Ontario replicate 2	0.018 (0, 0.77)	0.085 (0.009, 0.80)		
Central Ontario replicate 3	0.41 (0.34, 0.49)	0.70 (0.63, 0.77)		

[a]Values are mg acid equivalents/L; values in parentheses represent 95% confidence intervals.

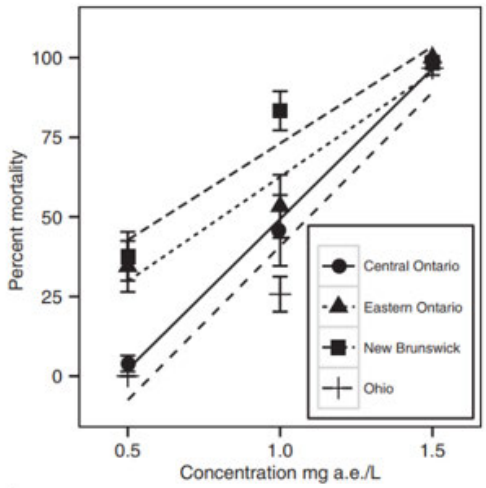


Figure 1 (from Edge C. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2628-32). Mean percent mortality (± standard error) of 4 populations of wood frog (*Lithobates sylvaticus*) tadpoles exposed to 5 concentrations of Roundup Weed and Grass Killer for 96 h. n = 6 for each concentration and population. a.e. = acid equivalents.

A similar difference in magnitude was observed among the 3 Central Ontario replicates. The LC_{50} values for Roundup Weed and Grass Control differed by 0.905 mg a.e./L, and the LC_{10} values differed by 0.71 mg a.e./L (Table 1). There were significant differences in mortality among the subpopulations ($p < 0.001$); mortality increased with exposure concentration, and the slopes of the response curves were significantly different ($p < 0.001$). In the first population, Ohio, exposed to Roundup WeatherMax, no mortality was observed in any of the exposure concentrations up to 4.0 mg a.e./L. Therefore, the exposure concentrations were doubled for the next population, Eastern Ontario. In the Eastern Ontario experiment, limited mortality was observed at concentrations up to 8.0 mg a.e./L. The exposure concentrations were doubled again for the New Brunswick population. In the New Brunswick exposures, some mortality was observed at concentrations at and above 4.0 mg a.e./L, but complete mortality in all replicates was observed only at the highest concentration, 16 mg a.e./L. Therefore, the researchers doubled the concentrations again for the last population, Central Ontario. In this experiment, mortality was observed at concentrations at and above 8.0 mg a.e./L, with complete mortality observed at concentrations at and above 16 mg a.e./L (Fig. 2). As such, the researchers can only crudely estimate that LC_{50} values for the Ohio and Eastern Ontario populations were greater than 4 mg a.e./L and 8 mg a.e./L respectively. In contrast, upward adjustment of the exposure concentration series allowed for the calculation of LC_{10} and LC_{50} values for the New Brunswick and Central Ontario populations (Table 1). Between populations, Roundup WeatherMax was more toxic to animals from New Brunswick than those from Central Ontario. The calculated LC_{10} and LC_{50} values indicate that the formulation Roundup Weed and Grass Control is approximately 5 times more toxic to amphibian larvae than Roundup WeatherMax.

Exposure validation

The mean percent deviation from the expected concentration was 1.2% (range, 0-9%). The largest deviations occurred in the Roundup WeatherMax exposure concentrations between 12 mg a.e./L and 32 mg a.e./L. Nominal target concentrations was used for all analyses.

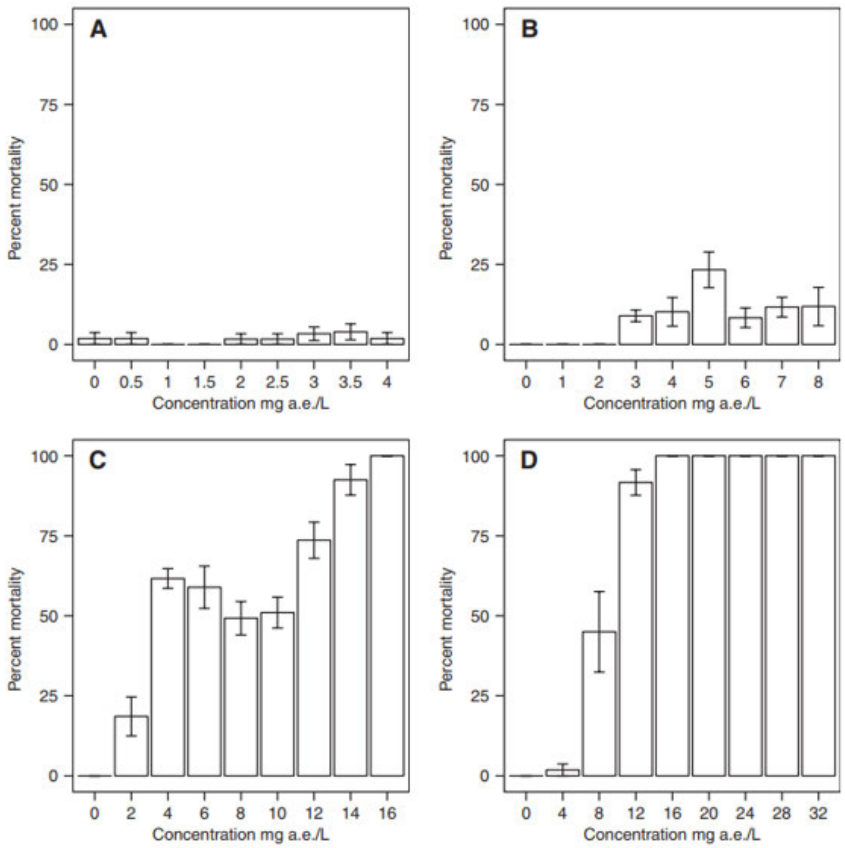


Figure 2 (from Edge C. *et al.* 2014, ET&C, Vol. 33, No. 11, pp. 2628-32). Mean percent mortality (± standard error) of 4 populations of wood frog (*Lithobates sylvaticus*) tadpoles, exposed to 9 concentrations of the herbicide Roundup Weathermax: (A) Ohio, USA; (B) Eastern Ontario, Canada; (C) New Brunswick, Canada; and (D) Central Ontario, Canada. n = 6 for each treatment and concentration. a.e. = acid equivalents.

Conclusions

The present study found that Roundup WeatherMax is approximately 5 times less toxic to larval wood frogs than Roundup Weed and Grass Control. This difference in toxicity is likely attributable to differences in surfactants or adjuvants among formulations. Although small but potentially meaningful differences in the toxicity of these herbicides among populations were observed, it is possible that these differences are due to among-experiment variations rather than differences in tolerance among the populations.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: None of the tested formulations (Roundup WeatherMax, Roundup Weed and Grass Control) is the representative formulation for the glyphosate EU renewal. Therefore, the article is not relevant for the renewal. The formulations Roundup Weed and Grass Control contains 5.18 g a.e./L in the form of the isopropylamine salt and the formulation Roundup WeatherMax contains 540 g a.e./L in the form of the potassium salt.

In addition, prior exposure history to other chemicals and other organisms within their natural environment was unknown. Several limitations were observed within the study including lack of exposure history of the local organisms, inability to attribute the results entirely to the test substance, inability to develop a dose-response relationship or derive end-points within the study, the analytical approach and verification was lacking, and the study was not conducted according to a standard guideline.

Moreover, this is a study which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

Differences in factors such as geoclimatic properties and land-uses etc are also not relevant in this context, as this is a laboratory study. Moreover, all eggs were collected from the same wetland and thus any potential impact of exposure history would also be observed in the control group. However, the authors state that, to their knowledge, the populations sampled had no prior exposure to herbicides. In addition, the argument that the analytical approach is lacking is inaccurate, since glyphosate was quantified using LC-MS, as also specified above in this summary.

The following endpoints were generated for *Lithobates sylvaticus* tadpoles:
Roundup Weed and Grass Control: 96 h LC_{50} ranging 0.59 to 1.10 mg a.e./L (geomean 0.65 mg a.e./L)
Roundup WeatherMax: 96 h LC_{50} ranging >4 to 8.26 mg a.e./L. (geomean 6.01 mg a.e./L)

The RMS considers this study to be reliable.

Data point	CA 9
Report author	Fuentes L. *et al.*
Report year	2011
Report title	Comparative toxicity of two glyphosate formulations (original formulation of Roundup® and Roundup WeatherMAX®) to six North American larval anurans
Document source	Environmental toxicology and chemistry (2011), Vol. 30, No. 12, pp. 2756-61
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The toxicity of two glyphosate formulations (the original formulation of Roundup® and Roundup WeatherMAX®) to six species of North American larval anurans was evaluated by using 96-h static, nonrenewal aqueous exposures. The 96-h median lethal concentration values (LC_{50}) ranged from 1.80 to 4.22 mg acid equivalent (ae)/L and 1.96 to 3.26 mg ae/L for the original formulation of Roundup and Roundup WeatherMAX, respectively. Judged by LC_{50} values, four species were more sensitive to Roundup WeatherMAX exposures, and two species were more sensitive to the original formulation. Two of six species, *Bufo fowleri* ($p < 0.05$, $F = 14.89$, degrees of freedom [*df*] = 1) and *Rana clamitans* ($p < 0.05$, $F = 18.46$, *df* = 1), had significantly different responses to the two formulations tested. Increased sensitivity to Roundup WeatherMAX likely was due to differences in the surfactants or relative amounts of the surfactants in the two formulations. Potency slopes for exposures of the original formulation ranged from 24.3 to 92.5% mortality/mg ae/L. Thresholds ranged from 1.31 to 3.68 mg ae/L, showing an approximately three times difference in the initiation of response among species tested. For exposures of Roundup WeatherMAX, slopes ranged from 49.3 to 84.2% mortality/mg ae/L. Thresholds ranged from 0.83 to 2.68 mg ae/L.

Materials and methods

Chemicals

The original formulation of Roundup® and Roundup WeatherMAX® were used in this study and were obtained from Monsanto. The original formulation of Roundup contained the isopropylamine (IPA) salt of glyphosate (29.7% a.e.) with MON 0818 (15%), a surfactant containing polyethoxylated tallow amine (POEA). Roundup WeatherMAX comprised the potassium salt of glyphosate (39.9% a.e.) with a proprietary surfactant. Acid equivalents were defined as the theoretical yield of the parent acid of the pesticide formulation.

Primary stock solutions for Roundup formulations were prepared at nominal concentrations of 1,000 mg ae/L using NANOpureTM water. The dilution water for the stock solution was formulated using compound and elemental ratios based on hardness and alkalinity ranges from water measurements throughout the United States; this water was also used for holding animals prior to testing.

Animals

Larval anuran amphibians at Gosner stage 25 are reported to be sensitive to exposures to Roundup formulations. Six species of anuran amphibians, bullfrog (*Rana catesbeiana*), green frog (*R. clamitans*), southern leopard frog (*R. sphenocephala*), northern leopard frog (*R. pipiens*), Fowler's toad (*Bufo fowleri*), and Cope's gray tree frog (*Hyla chrysoscelis*) were selected for laboratory testing because of the coincidence of their breeding seasons with agricultural or silvicultural herbicide applications in North America. *Rana sphenocephala*, *R. clamitans*, *B. fowleri*, and *H. chrysoscelis* eggs were obtained from local collections from Pickens and Greenwood Counties in South Carolina, USA, and *R. catesbeiana* and *R. pipiens* eggs were purchased from vendors. Eggs were held in the Clemson University Aquatic Animal Research Laboratory in 38-L glass tanks with no more than 300 eggs/L per tank. Animals were fed ad libitum twice daily. Holding chambers were cleaned daily, and approximately half of the water was renewed every other day. Temperature, light quality, light intensity, photoperiod, and aeration were maintained within recommended ranges throughout laboratory holding and testing periods. In addition, water characteristics were kept within recommended ranges and similar to conditions in which egg masses were located. To initiate a toxicity test, organisms were allowed to develop to approximately Gosner stage 25. Animals were eligible for testing if observations of external appearance and behavior indicated no signs of disease or death, discoloration, or unusual behavior according to the National Research Council's approved guidelines. Populations were considered suitable for testing if mortality did not exceed 10% during the holding period. All standard operating procedures were approved by the Clemson University Institutional Animal Care and Use Committee.

Experimental design

Toxicity tests were static, nonrenewal exposures, and animals were not fed during testing to maintain water quality. The 96-h exposure period was selected because it was of sufficient duration to capture the responses of amphibians to acute exposures and represents an environmentally realistic duration of exposure. For herbicide exposures, a stock solution of each formulation was prepared along with at least

10 nominal exposure concentrations with four replicates per concentration. A cosolvent was not used because the solubility of glyphosate and the surfactant greatly exceeded the concentrations used in these experiments. Testing vessels (replicates) were 3.8-L glass jars filled with 3 L of test solution. Exposure concentrations of both formulations were arrayed according to range-finding tests and were between 0.3 and 7.0 mg ae/L. This range of concentrations covers both ecologically relevant concentrations and concentrations intended to saturate responses. Prior to adding the animals, a sample was taken from each testing vessel for concentration verification. Ten tadpoles were exposed in each replicate, and the number of surviving animals was recorded at 24, 48, 72, and 96 h. An untreated control (no herbicide added) was established with four replicates and 10 animals per testing vessel to represent unexposed conditions. The mortality endpoint was indicated by failure to respond to gentle prodding stimuli from a blunt instrument.

Sample verification
Prior to adding the animals at the initiation of a test, water samples were taken from each testing vessel, stock solution, and dilution water and placed into silanized glass vials for analytical concentration verification. Samples were placed in cold storage at 3°C until time of analysis. All samples were derivatized, then analyzed for glyphosate based on a standard curve using a Dionex, UltiMate-3000 high-performance liquid chromatography (HPLC) system with a variable wavelength detector and auto sampler. Reagents included 0.37 M potassium tetraborate, 0.025 M methanolic NBD-CL (4-chloro-7-nitrobenzofurazan), and 1.2 M hydrochloric acid. Glyphosate analytical standard (99.8% purity) for preparation of calibration standards was provided by Monsanto. Samples were eluted with HPLC-grade acetonitrile and 0.1% phosphoric acid. The column used was a YMC-Pack reversed-phase (octadecylsilane, ODS-AM series) high-carbon-load packing material with a 40-ml injection volume and a primary wavelength of 500 nm. Results are reported with a range of 85 to 115% recovery according to external standards. Because the nominal concentrations of glyphosate were analytically verified in each replicate at all concentrations tested, the mathematical mean concentration of the four replicates at each concentration was used to run statistical analyses and report results.

Data analysis
Data were analyzed using SAS1 software version 9.1. Not all mortality data met the assumptions for parametric analysis. When appropriate (normally distributed data), probit regression (PROC PROBIT) was used to determine no-observed-effect concentrations (NOEC), lowest-observed-effect concentrations (LOEC), lethal concentration values (LC_x), and 95% confidence intervals (CI). The U.S. Environmental Protection Agency MS-DOS application for trimmed Spearman-Karber analysis was used to obtain LC_{50} values and 95% CI for nonparametric data. In addition, nonparametric rank converted analyses of variance with Dunnett's test analyses were used to determine NOEC and LOEC values for non-parametric data. A NOEC value was designated as the last exposure concentration not significantly different from zero based on 95% CI, above which the next concentration that was significantly different from zero was designated as the LOEC value. Lethal concentration values were considered significantly different from each other when their 95% CI did not overlap. To make comprehensive comparisons between concentration–response relationships as a function of formulation type and species, threshold and slope were evaluated. The linear portion of the potency curve, from approximately the NOEC to the first concentration resulting in 100% mortality was used to generate the regression equation containing the potency slope and threshold. This regression equation was produced using PROC REG. An R^2 value greater than or equal to 0.80 was considered to be an acceptable ratio of explained variation in the generated model. Potency slopes were compared using one-way analysis of covariance with a general linear model (PROC GLM).

The slopes of concentration–response data were calculated to compare the rate of change of response (potency) for the species tested. The rate of change indicated the magnitude of effect of the concentration on toxicity. Threshold values were estimated by averaging the NOEC and LOEC that estimates initiation of the increase in percent mortality with increasing concentration of herbicide. Slope values are expressed as percentage mortality/mg ae/L.

Results

Quality control measures

Control mortality did not exceed 7.5% for any laboratory test. Prior to testing, initial water characteristics were measured. Water conductivity averaged 156.0 µs/cm^2 ± 8.3 (range 144.1–179.0). The mean dissolved oxygen was 8.1 mg/L ± 0.9 (5.2–9.5). The initial pH of dilution water averaged 7.5 ± 0.2 (6.7–7.7). Hardness and alkalinity averaged 35 mg $CaCO_3$/L ± 4.3 (26–44) and 41 mg $CaCO_3$/L ± 3.4 (34–50), respectively. Temperature remained at 20 ± 1°C throughout holding and testing periods.

Ninety-six-hour LC_{50}s

Judged from 95% CI, all species tested had significantly different responses between the two formulations (Table 1). *Rana pipiens* was the most sensitive to exposures of the original formulation of Roundup; *R. clamitans* was the least sensitive. Ninety-six-hour LC_{50} values of the six species ranged from 1.80 to 4.22 mg ae/L for all species exposed to the original formulation. Among the species tested, *Rana sphenocephala* was sensitive to exposures of Roundup WeatherMAX, and *H. chrysoscelis* was insensitive, with 96-h LC_{50} ranging from 1.33 to 3.26 mg ae/L for all species tested. Four of the six species tested were less sensitive to exposures of the original formulation compared with exposures of Roundup Weather-MAX based on 96-h LC50 values (Table 1). *Rana pipiens* and *H. chrysoscelis* were the two species that were less sensitive to exposures of Roundup WeatherMAX compared with exposures of the original formulation.

Table 1 (from Fuentes L. *et al.* 2011, ET&C, Vol. 30, No. 12, pp. 2756-61). Responses of six larval anuran species to the original formulation of Roundup® and Roundup® WeatherMAX (Monsanto, USA) in 96-h aqueous.

Species	96-h LC50[a]	NOEC[a]	LOEC[a]	Slope[b]	Threshold	Margin of safety[c]
Original formulation of Roundup (mg acid equivalent/L of glyphosate)						
Rana pipiens	1.80 (1.73–1.88)	1.29 (1.16–1.39)	1.32 (1.19–1.41)	92.5	1.31	1.4/23
R. sphenocephala	2.05 (1.90–2.20)	1.52	1.81	47.9	1.67	1.7/28
Hyla chrysoscelis	2.50 (2.38–2.63)	1.74	2.10	44.7	1.92	1.9/32
R. catesbeiana	2.77 (2.66–2.89)	2.02	2.52	66.9	2.27	2.2/37
Bufo fowleri	4.21 (4.08–4.33)	3.40	3.95	47.2	3.68	3.7/62
R. clamitans	4.22 (4.02–4.42)	3.27	3.68	24.3	3.48	3.6/59
Roundup WeatherMAX (mg acid equivalent/L of glyphosate)						
R. sphenocephala	1.33 (1.22–1.45)	0.68	0.98	54.7	0.83	0.8/12
B. fowleri	1.96 (1.89–2.04)	1.54 (1.40–1.64)	1.56 (1.42–1.66)	84.2	1.55	1.7/27
R. catesbeiana	1.97 (1.89–2.06)	1.33 (1.16–1.46)	1.37 (1.20–1.49)	63.0	1.35	1.5/23
R. pipiens	2.27 (2.18–2.36)	1.65 (1.48–1.77)	1.68 (1.52–1.80)	65.5	1.67	1.8/29
R. clamitans	2.77 (2.67–2.87)	1.91	2.37	62.2	2.14	2.1/34
H. chrysoscelis	3.26 (3.14–3.38)	2.48	2.87	49.3	2.68	2.7/44

[a] Median lethal concentration (LC50) no-observed-effect concentration (NOEC), and lowest-observed-effect concentration (LOEC) with 95% confidence intervals provided if available through probit analysis.
[b] Values expressed as percentage mortality/mg acid equivalents/L.
[c] Based on predicted environmental concentration for the median label application rate assuming a direct overspray/5% drift, and a water depth of 15.34 cm (6 inches).

Potency slope and threshold

Potency slopes for exposures of the original formulation of Roundup ranged from 24.3 to 92.5% mortality/mg ae/L. *Rana clamitans* had the least precipitous slope, and *R. pipiens* showed the greatest change in response with increasing concentration. Thresholds ranged from 1.31 to 3.68 mg ae/L, showing an approximately three times difference in the initiation of response between *R. pipiens* and *B. fowleri*. For exposures of Roundup WeatherMAX, slopes ranged from 49.3 to 84.2 for *H. chrysoscelis* and *B. fowleri*, respectively. Thresholds ranged from 0.83 to 2.68 mg ae/L for *R. sphenocephala* and *H. chrysoscelis*, respectively (Table 1 and Fig. 1). Analysis of covariance indicated that two of the six species, *B. fowleri* ($p < 0.05$, $F = 14.89$, $df = 1$) and *R. clamitans* ($p < 0.05$, $F = 18.46$, $df = 1$), had significantly different rates of response between the two formulations.

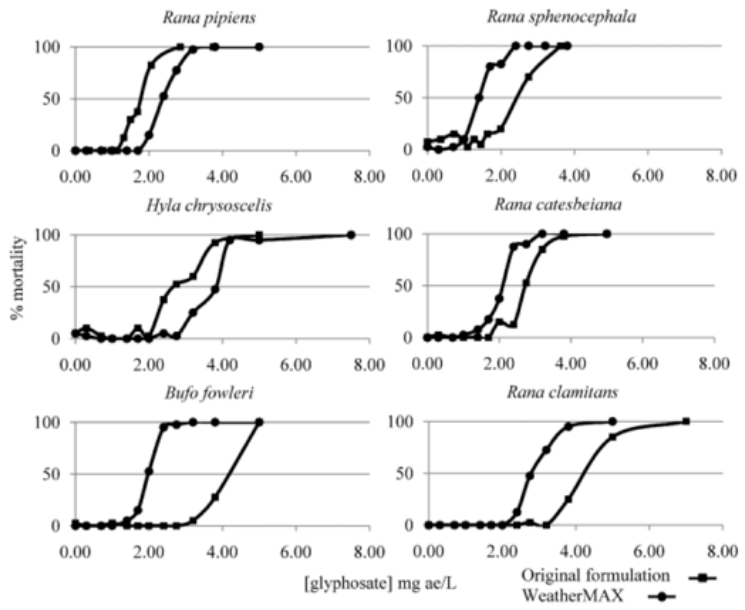


Figure 1 (from Fuentes L. *et al.* 2011, ET&C, Vol. 30, No. 12, pp. 2756-61). Concentration–response curves of six species of larval anurans to 96-h aqueous exposures of the original formulation of Monsanto Roundup® (■) and Roundup WeatherMAX® (●).

Conclusion

The experimental design of this study, with close spacing of environmentally relevant test concentrations, allows exposure-response relationships to be determined with greater accuracy than in most acute toxicity tests. These data can be used to assess the risk to anurans from exposures to the two formulations of Roundup. Based on the generated NOEC values for the species tested, with the exception of *R. sphenocephala* when exposed to Roundup WeatherMAX, all species had an potential margin of safety (MOS) > 1 for the original formulation of Roundup and Roundup WeatherMAX, which indicated little risk from exposures at the respective PECs. The MOS values are dependent on proper usage in accordance with product labels.

For Roundup® the 96 hour LC_{50} values of the six species ranged from 1.80 to 4.22 mg ae/L. Among the species tested, *Rana sphenocephala* was sensitive to exposures of Roundup WeatherMAX, and *H. chrysoscelis* was less sensitive, with 96-h LC_{50} ranging from 1.33 to 3.26 mg ae/L for all species tested. Four of the six species tested were less sensitive to exposures of the original formulation Roundup® compared with exposures of Roundup Weather-MAX based on 96 hour-LC_{50} values. *Rana pipiens* and *H. chrysoscelis* were the two species that were less sensitive to exposures of Roundup WeatherMAX compared with exposures of the original formulation.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The original formulation of Roundup and Roundup WeatherMAX are not the representative formulation for the glyphosate EU renewal. Although Roundup formulation was tested, it does however contain different surfactant system than the representative formulation MON 52276 for the glyphosate EU renewal. The surfactant system used in the formulation tested in this paper is polyethoxylene amine based (POEA), whereas the surfactant used in MON 52276 is quaternary-ammonium based. POEA surfactant is not permitted in formulated herbicidal products in the EU. As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal. The test design is well described in the paper, but due to the test materials not being the representative formulation for the glyphosate EU renewal, the study is considered not relevant.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Regarding the tested original Roundup formulation, the results are not considered relevant for the assessment of the representative product since the tested formulation includes surfactants that are similar to substances not allowed in the EU (Regulation (EU) 2016/1313 and/or Regulation (EU) 2021/383.

However, Roundup WeatherMAX contains an unspecified surfactant, and thus there is not sufficient information in the study report to demonstrate whether this formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The 96 h LC_{50} values for amphibians ranged 1.33-3.26 mg a.e./L.

The RMS considers this study to be reliable.

Data point	CA 9
Report author	Jones D. K. *et al.*
Report year	2011
Report title	Competitive stress can make the herbicide Roundup® more deadly to larval amphibians
Document source	Environmental Toxicology and Chemistry (2011), Vol. 30, No. 2, pp. 446-454
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

To explore how the natural stress of competition might interact with a glyphosate-based herbicide, the researchers used outdoor mesocosms containing three tadpole species that were exposed to a factorial combination of three glyphosate concentrations (0, 1, 2, or 3 mg acid equivalent (a.e.)/L of the commercial formulation Roundup Original MAX®) and three tadpole densities (low, medium, or high). They found that increased tadpole density caused declines in tadpole growth, but also made the herbicide significantly more lethal to one species. Whereas the median lethal concentration (LC_{50}) values were similar across all densities for gray treefrogs (*Hyla versicolor*; 1.7-2.3 mg a.e./L) and green frogs (*Rana clamitans*; 2.2-2.6 mg a.e./L), the LC_{50} values for bullfrogs (*R. catesbeiana*) were 2.1 to 2.2 mg a.e./L at low and medium densities, but declined to 1.6 mg a.e./L at high densities. The large decrease in

amphibian survival with increased herbicide concentration was associated with increases in periphyton abundance. The researchers also found evidence that temperature stratification lead to herbicide stratification in the water column, confirming the results of a previous study and raising important questions about exposure risk in natural systems.

Materials and methods

The main focus of the study was to evaluate the impact of various concentrations of glyphosate on tadpole (Roundup Original MAX®) survival within a simulated outdoor environment including differing amounts of tadpole density.

The effects of Roundup Original MAX® and tadpole density on wetland communities was evaluated. An outdoor mesocosm experiment at the University of Pittsburgh's Pymatuning Laboratory of Ecology in northwestern Pennsylvania (USA) was conducted. The experiment employed a randomized block design consisting of 12 treatments that were replicated twice within each of two spatial blocks for a total of 48 experimental units. The 12 treatments represented a factorial combination of four concentrations of the herbicide (0, 1, 2, or 3 mg a.e./L of glyphosate) crossed with three tadpole densities (low, medium, or high).

The mesocosms were 750-L round, cattle tanks filled with approximately 562 L of well water (pH 8) Water depth was approximately 0.4 m. All mesocosms also received equal aliquots of water, collected and pooled from multiple nearby ponds, to create seminatural pond communities consisting of periphyton, phytoplankton, and zooplankton. Invertebrate predators were removed prior to adding the pond water to the mesocosms. A single ceramic tile was placed into each tank to serve as a periphyton sampler. Mesocosms were covered with 60% shade cloth to prevent other organisms from ovipositing, and were allowed to develop algal and invertebrate populations for two weeks prior to adding the amphibians.

The amphibians used in this experiment were collected as egg masses from nearby ponds. American bullfrogs (*Rana catesbeiana*; collected on May 26 through June 4), 15 clutches of green frogs (*R. clamitans*; collected on May 26 through 28), and 14 clutches of gray tree frogs (*Hyla versicolor*; collected on May 26 through 29). The eggs were hatched in covered wading pools containing aged well water. Several hundred hatchlings were raised together, with water changes every few days once the animals became large enough to safely handle. Amphibian larvae were added to the mesocosms on July 2 and 3 (defined as days 0 and 1 of the experiment).

Tadpole density (i.e., competition) was manipulated by adding 20, 40, or 60 tadpoles of green frog and gray tree frog tadpoles to each mesocosm. Due to a limited number of bullfrog tadpoles, all mesocosms received an equal number of bullfrogs (20 per tank).

On day 7, the herbicide treatments were applied. Based on an estimate of 562 L of water in each mesocosm, amounts of 0, 1.041, 2.082, and 3.124 ml of Roundup Original MAX® formulation to obtain nominal glyphosate concentrations of 0, 1, 2, and 3 mg a.e./L (reported to contain 48.7% glyphosate) was added. The analysis of the water samples confirmed that the herbicide stratified in the water column. Glyphosate concentrations collected near the surface were higher than nominal, whereas concentrations near the bottom of the mesocosm were lower than nominal. However, the means of the top and bottom values were very close to the nominal concentrations.
Water quality measurements of temperature, pH, and dissolved oxygen were measured twice (on days 7 and 16). To determine whether the water experienced stratification, temperature and pH values were measured at the top and bottom of the water column. Dissolved oxygen was taken in the middle of the water column. Periphyton biomass was assessed on day 15.

Statistical Tests
To determine how tadpole survival was affected by glyphosate concentration and tadpole density, data analyses were conducted using multivariate analysis of variance (MANOVA).

Initial analyses indicated that none of the species exhibited significant block effects or block interactions, so these terms were dropped from the analysis and the degrees of freedom were pooled with the error term. Most assumptions of the analysis were met, with the exception of homoscedastic variance. However, analyses of variance are generally robust to the violation of this assumption. To quantify the LC_{10}, LC_{50}, and LC_{90} values (the lethal concentration expected to kill 10, 50, and 90% of a population), the researchers conducted standard probit analyses for each tadpole species within each level of competition. For each density, they adjusted for the low amounts of mortality in the controls using the procedures recommended by Finney (1971). Significant differences in estimates can be determined by nonoverlapping 84% confidence intervals; simulation studies find that this confidence interval approximates an $\alpha = 0.05$.

Results

The MANOVA on tadpole survival found significant effects of glyphosate concentration and indicated marginal effects of tadpole density and the glyphosate-by-density interaction.

Gray tree frog survival (Fig. 1) was considered affected by glyphosate concentration, but not by tadpole density or their interaction (Fig. 2). The LC_{50} values for gray tree frogs across the three density treatments ranged from 1.7 to 2.3 mg a.e./L.

Green frog survival (Fig. 1) was affected by glyphosate concentration, marginally by tadpole density, but not their interaction (Fig. 2). The LC_{50} values for green frogs across the three density treatments ranged from 2.2 to 2.6 mg a.e./L.

Bullfrog survival (Fig. 1) was affected by glyphosate concentration, the glyphosate-by-density interaction, and marginally by tadpole density (Fig. 2). The LC_{50} values for bullfrogs reflected this competition effect; LC_{50} values were similar at low and medium densities (2.1 to 2.2 mg a.e./L), but both were significantly different from the LC_{50} values at high tadpole density (1.6 mg a.e./L).

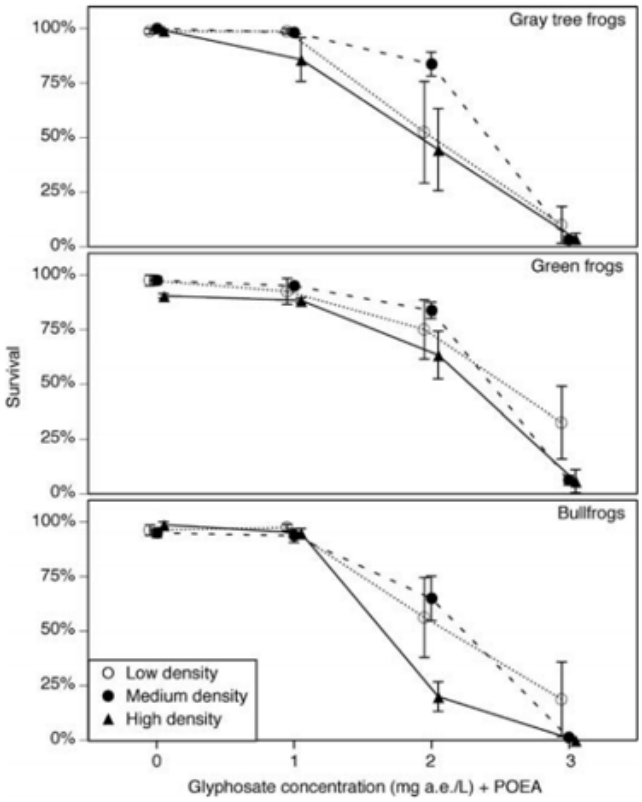


Figure 1 (from Jones D. K. *et al.* 2011, ET&C, Vol. 30, No. 2, pp. 446-454). Survival of three species of tadpoles when exposed to three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX®, Monsanto). Data points represent means one standard error. To make

the different error bars clearer, the low and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

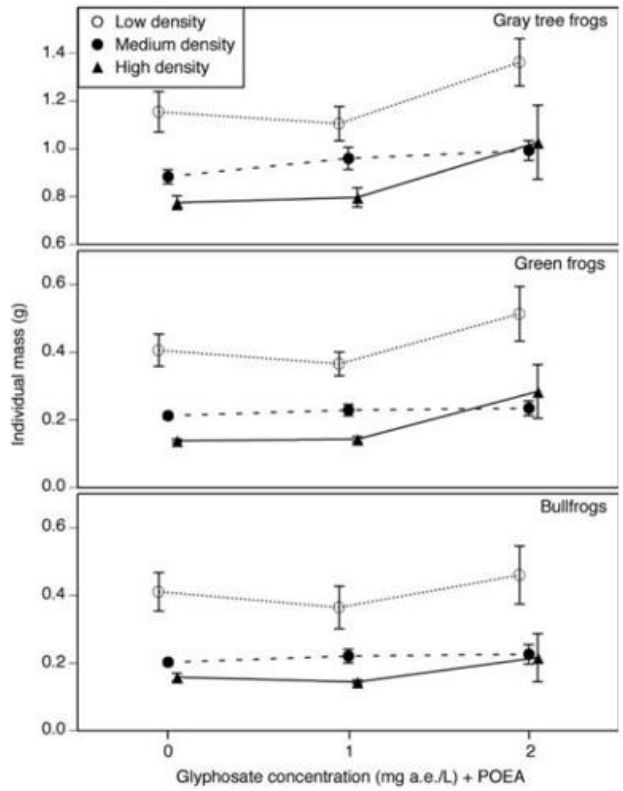


Figure 2 (from Jones D. K. *et al.* 2011, ET&C, Vol. 30, No. 2, pp. 446-454). Individual mass of three tadpole species when exposed to three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX®, Monsanto). Data points represent means one standard error. To make the different error bars clearer, the low- and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

Periphyton biomass (Fig. 3) was significantly affected by glyphosate concentration ($F_{3,24}$ = 17, $p < 0.001$), but not by tadpole density ($F_{2,24}$ = 0.7, $p = 0.50$) nor their interaction ($F_{6,24}$ = 1.6, $p = 0.20$; Fig. 3). Mean comparisons indicated that glyphosate concentrations of 0, 1, and 2 mg a.e./L had similar amounts of periphyton biomass ($p > 0.62$), whereas 3 mg a.e./L was associated with nearly twice as much periphyton ($p < 0.001$).

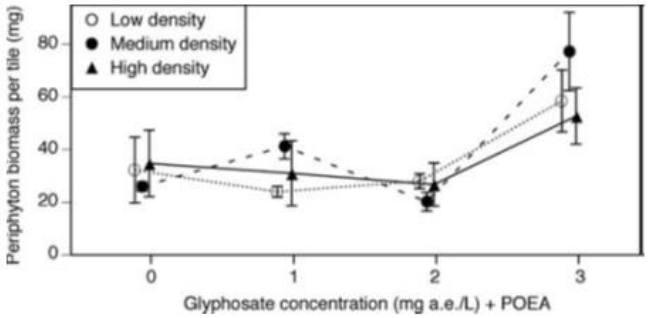


Figure 3 (from Jones D. K. *et al.* 2011, ET&C, Vol. 30, No. 2, pp. 446-454). Periphyton abundance on day 15 of the experiment across three tadpole densities and four concentrations of glyphosate (applied as the commercial formulation of Roundup Original MAX®, Monsanto). Data points represent means one standard error. To make the different error bars clearer, the low- and high-density values are shifted slightly along the x axis. a.e. = acid equivalent; POEA = polyethoxylated tallowamine.

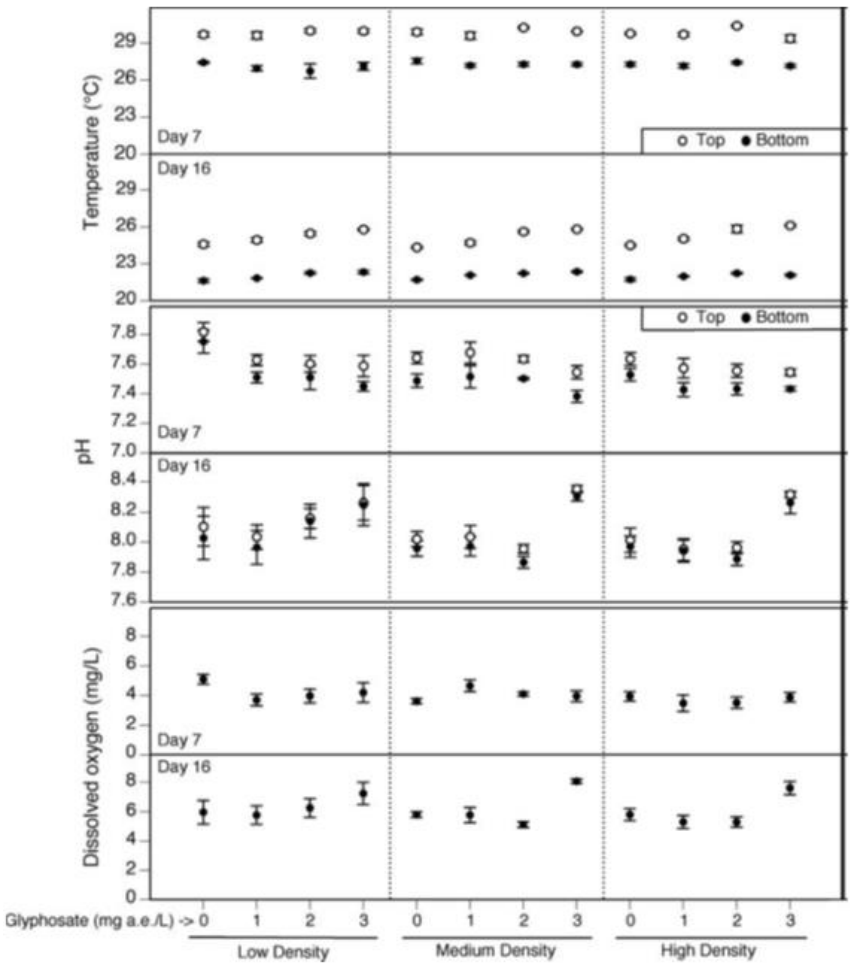


Figure 4 (from Jones D. K. *et al.* 2011, ET&C, Vol. 30, No. 2, pp. 446-454). Abiotic conditions of the mesocosm experiment; temperature and pH of mesocosms taken on days 7 and 16, assayed at the top and bottom of the water column. Dissolved oxygen measurements were taken in the middle of the water column. Data points represent means one standard error. a.e. = acid equivalent.

The examination of contaminant impacts under single-species laboratory conditions is an excellent first step in understanding the impact that contaminants could have on nontarget organisms. The present study highlights the new insights that can be gained by placing nontarget organisms into more natural conditions in which individuals can interact and temperature stratification can occur. Although the lethality of Roundup Original MAX was consistent with past laboratory and mesocosm experiments, the present study also identified that competition made this formulated product significantly more lethal to one species of tadpoles. Future work should examine the generality of this outcome, both by examining additional species of tadpoles and by determining the underlying mechanism. Given that several different biotic stressors can make a diversity of animals more susceptible to a range of pesticides, a common mechanism may underlie all of these studies.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: This study assessed competition as a stressor in conjunction with Roundup Original MAX® treatment in an outdoor mesocosm (USA) containing different densities of tadpoles (green frogs, gray tree frogs, american bullfrogs). Glyphosate product was applied up to 3 mg a.e./L for 7 days with replication. Egg masses were collected from nearby ponds and hatched in wading pools with aged well-water. Due to the test materials not being the representative formulation for the glyphosate EU renewal, the study is not relevant.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The LC_{50} values for Roundup Original Max, based on the 'low competition' treatments are:
2.18 mg a.e./L for *Rana catsbeiana*
2.04 mg a.e./L for *Hyla versicolor*
2.58 mg a.e./L for *Rana clamitans*

The RMS considers the study to be reliable.

Data point	CA 9
Report author	Jones D. K. *et al.*
Report year	2010
Report title	Roundup and amphibians: the importance of concentration, application time, and stratification
Document source	Environmental toxicology and chemistry (2010), Vol. 29, No. 9, pp. 2016-25
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The researchers examined the role of application amount, timing, and frequency using outdoor mesocosm communities containing larval amphibians (*Rana sylvatica* and *Bufo americanus*) and using a commercial formulation of the herbicide glyphosate (Roundup Original MAX®). Consistent with past studies, exposures of up to 3 mg acid equivalent (a.e.)/L caused substantial amphibian death. However, the amount of death was considerably higher when the herbicide was applied earlier in the experiment than later in the experiment. Single, large applications (at different times) had larger effects on tadpole mortality and growth than multiple, small applications (of the same total amount). The results may reflect an acclimation to the herbicide over time. In treatments with high tadpole mortality, there was no resulting increase in periphyton, suggesting that the reduction in tadpole herbivory might have been offset by direct negative impacts of the herbicide. The researchers also discovered that temperature stratification caused herbicide stratification, with higher concentrations near the surface. Such stratification has important implications to the habitat choices of ectotherms that might prefer surface waters for thermoregulation or prefer deeper waters to avoid predators. Collectively, the present study

demonstrates the importance of examining multiple applications times and frequencies to understand the impacts of pesticides on organisms.

Materials and methods

The formulated product that was used in this study, Roundup Original MAX®, is a popular formulation for agricultural applications. Roundup Original MAX® contains 48.7% glyphosate active ingredient (a.i.) plus inert ingredients that include a surfactant. Although the surfactant is reportedly not POEA (S. Mortensen, Monsanto, personal communication), past research has determined that the toxicity of this formulation to larval amphibians is nearly identical to other glyphosate formulations that do contain POEA.

The mesocosm experiment
To examine the importance of glyphosate concentration and application time on larval amphibians, the researchers conducted an outdoor mesocosm experiment at the Pymatuning Laboratory of Ecology in Pennsylvania. Mesocosm experiments represent seminatural conditions and this experiment exposed two species of spring-breeding larval amphibians (from two families: wood frogs, *Rana sylvatica* [Ranidae] and American toads, *Bufo americanus* [Bufonidae]) to 12 different herbicide treatments in a completely randomized design. The first treatment was a no-pesticide control. The next nine treatments examined three concentrations of glyphosate (1, 2, and 3 mg a.e./L) that were applied once on either day 0, day 7, or day 14. The final two treatments applied glyphosate (0.33 or 1.00 mg a.e./L) repeatedly at three time points (on days 0, 7, and 14). In this case, the researchers simulated events in which a wetland was contaminated on three occasions during a 3-week period, a scenario that could happen following accidental oversprays or drift combined with rain events that wash the herbicide off plant surfaces and into the water body. Any tanks not receiving glyphosate on days 0, 7, or 14 were mock-dosed to equalize disturbance. The 12 treatments were replicated four times for a total of 48 mesocosms.

The mesocosms were 750-L cattle tanks, filled with approximately 542 L of well water on April 28-29, 2007. To each mesocosm, we added 15 g of rabbit chow as an initial nutrient source, 200 g of oak (*Quercus* spp.) leaf litter to serve as a natural substrate for periphyton growth, and two aliquots of local pond water containing zooplankton, phytoplankton, and periphyton.

Two ceramic tiles (15x15 cm) were placed into each tank to assess periphyton growth. Mesocoms were allowed 2 weeks to develop algal and zooplankton communities before the amphibians were introduced. All mesocosms were covered with 60% shade cloth lids to prevent other organisms from ovipositing into the water.

The wood frogs and American toads were collected as egg masses from nearby ponds (20 clutches of wood frogs and 10 clutches of American toads) and hatched in covered culture pools filled with aged well water. The hatchlings were fed rabbit chow *ad libitum* until used in the experiment. Once both tadpole species became large enough to safely handle, the researchers mixed the hatchlings of all clutches and selected tadpoles for the experiment from this mixture. Both species of tadpoles were early in development. The initial developmental stage and mean mass (± 1 standard error) was stage 26 and 106 ± 7mg for wood frogs and stage 25 and 17 ± 2 for American toads. Every tank received 20 wood frog tadpoles and 20 American toad tadpoles. This density, 8 individuals/m^2, is well within natural levels (R. A. Relyea, personal observation). Following the addition of both tadpole species on May 18, 2007 (defined as day 0), the researchers applied the herbicide treatments. To obtain nominal concentrations of 0, 0.33, 1, 2, and 3 mg a.e./L of glyphosate, the researchers added 0, 0.333, 1, 2, and 3 mL of Roundup Original MAX.

The herbicide was dissolved into a half liter container of water that was then drizzled across the surface of the water. The surface water was then mixed to help distribute the herbicide. Approximately 3 h after dosing, water samples were taken to determine the actual concentration of glyphosate in the water and to determine whether the concentrations of the herbicide differed between the top and bottom of the water column. The researchers took water samples from the four replicates of each treatment from both the top and bottom of the water column using a tube sampler. Water samples from the four replicate

tanks were pooled and placed into a pre-cleaned, glass amber jar and held at 28 °C to prevent breakdown. Water from the control (0 mg a.e./L) treatments was not tested, but past well water tests have found no traces of glyphosate. The tested water samples indicated that the herbicide concentrations differed between the top and bottom of the water column. For all treatments receiving single applications on day 0, the mean observed concentrations from the top and bottom water samples were very close to the target concentrations and, for simplicity, will be referred to as 0, 0.33, 1, 2, and 3 mg a.e./L.

Later single applications were assumed to have the same concentrations as early single applications of the same amount and thus were not tested. For treatments receiving multiple applications of glyphosate, a second and third set of samples were tested to determine if the second and third applications caused an accumulation of glyphosate. These samples indicated that the three applications accumulated to a concentration that was 70 to 87% of the nominal concentration, thus demonstrating relatively little herbicide breakdown.

Water quality and periphyton
Water quality measurements were taken to assess the abiotic conditions of the experiment. Temperature and pH were measured at the top and bottom of the mesocosm (5 cm below the water's surface and 5 cm above the bottom of the tank, respectively). Dissolved oxygen was taken midway in the water column. Water quality measurements were taken twice during the experiment (before glyphosate application on day 7 and day 14) using a multimeter probe that was calibrated before each use (Multiline P4 Universal Meter, WTW). To obtain insight into periphyton abundance over time, the periphyton was assessed on day 12 and day 17. Ceramic tiles were removed from mesocosm tanks, scrubbed for periphyton, and rinsed with filtered well water. The resulting slurry was vacuum-filtered through a pre-dried (808 °C, 24 h) and pre-weighed Whatman 1 GF/C filter.

The experiment was terminated on day 18, because the toads were about to initiate metamorphosis and lose considerable mass. Amphibians were euthanized with MS-222 and then preserved in 10% formalin. Preserved animals were later counted and weighed to determine the survival and growth of the two species. The survival of each species from each tank and the mean mass of individuals from each mesocosm served as the amphibian response variables. The average developmental stage of the tadpoles at the end of the experiment was 38.2 for toads and 37.8 for wood frogs.

Statistical analysis
The researchers used analysis of variance to determine the effects of application amount and timing on the survival and mass of wood frogs and American toads. The researchers began by conducting a multivariate analysis of variance (MANOVA) across all 12 treatments to determine if there was a significant multivariate effect of the treatments. Following a significant multivariate effect, the researchers conducted separate univariate analyses of variance (ANOVAs) on the four response variables (wood frog survival and mass, toad survival and mass). For significant univariate effects, the researchers tested several hypotheses using planned mean comparisons. To determine the lowest observable-effect concentration (LOEC), the researchers used a Dunnett test that compared the control treatment to each treatment containing glyphosate. To determine whether multiple small applications differed from single, larger applications at three different times, the researchers conducted Tukey mean comparison tests.

The researchers also estimated the concentrations that would cause 10, 50 and 90% mortality (LC_{10}, LC_{50}, and LC_{90}, respectively) for each species at each application time using standard probit analyses.

The survival and mass data were approximately normal without having to be transformed. A few of the treatments had low variance (e.g., control wood survival 100% toad survival 98%) or high variance (e.g., toad mass early application 3 mg a.e./L), but ANOVAs tend to be robust to violations of this assumption. For toad mass, the variance for the early 3 mg a.e./L was substantially greater than all other treatments, likely due to higher death and, hence, fewer individuals and replicates. The analysis was run both with and without this treatment and the interpretation of the results was not altered. Thus, the researchers left the treatment in the analysis. The researchers chose not to rank-transform the data because doing so dramatically altered the relative survival values in a way that obfuscated obvious interactive treatment

effects. To test if concentration and application time had interactive effects on tadpole survival and mass, the researchers analyzed a subset of the data that only contained the three concentrations (1, 2, or 3 mg a.e./L) that were applied at three different times (day 0, 7, or 14). The researchers with a MANOVA followed by four ANOVAs to test the main effects and concentration-by-application time interactions. The survival and mass data for this subset of treatments generally met the assumptions of normality and equality of variance. Significant ANOVAs were followed by mean comparisons using a Tukey mean comparison test. Measurements of periphyton and water quality were separately analyzed with repeated-measures ANOVAs (rm-ANOVAs) since they were measured on multiple days. For variables that were measured at two water depths (pH and temperature at the top and bottom of the mesocosm), the data were analyzed by rm-ANOVAs while nesting sample depth within each treatment. When treatment effects were detected, planned contrasts were conducted to compare all treatments to the control. The data were log-transformed to achieve normality and homogeneity of variances.

Results

Amphibian survival and growth

There was a significant multivariate effect of the 12 treatments on amphibian survival and mass (Wilks' $F_{44,117} = 8.1$, $p < 0.0001$). Survival in the control treatment was high for both wood frogs (100%) and toads (98%). For wood frogs, survival was reduced with early applications of 2 or 3 mg a.e./L, midway applications of 2 or 3 mg a.e./L, and late applications of 3 mg a.e./L. Similarly, for American toads, survival was reduced with early applications of 2 or 3 mg a.e./L, midway applications of 3 mg a.e./L, and late applications of 3 mg a.e./L. Thus, Roundup's impacts on tadpole survival varied with application time and concentration. Next, the researchers quantified the LC_{10}, LC_{50}, and LC_{90} values to determine whether the values differed (based on non-overlapping LC_{50} 84% confidence intervals). For both species of tadpoles, the LC_{50} estimates for early and midway applications were similar to each other, but significantly lower than the LC_{50} estimates for late applications.

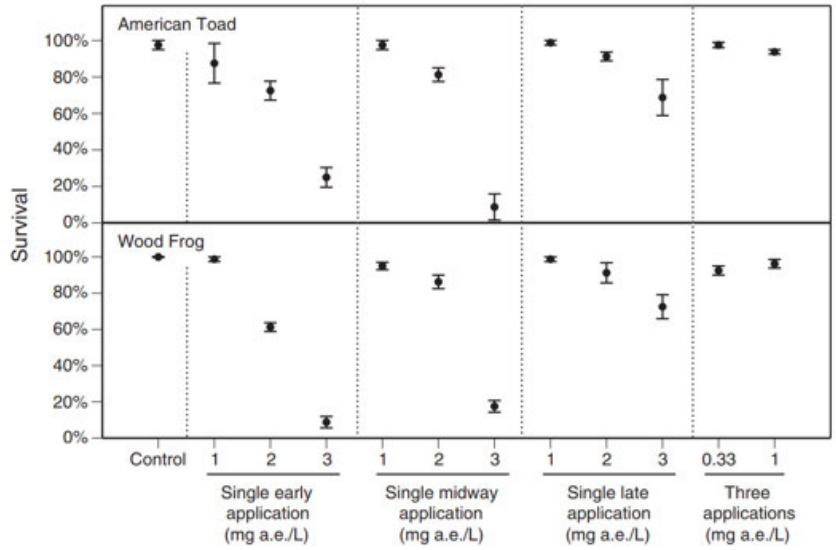


Figure 1 (from Jones D. K. *et al.* 2010, ET&C, Vol. 29, No. 9, pp. 2016-25). Survival of American toad and wood frog tadpoles when exposed to varying Roundup Original MAX® concentrations (mg a.e. of glyphosate/L) at different times (day 0, 7, or 14). Data points represent mean survival (1 standard error) for all four replicates. Survival was recorded on day 18 following experimental takedown.

Table 1 (from Jones D. K. *et al.* 2010, ET&C, Vol. 29, No. 9, pp. 2016-25). Results of probit analyses used to estimate the LC_{10}, LC_{50}, and LC_{90} values (lethal concentrations that cause 10, 50, and 90% mortality) for Roundup Original MAX® in outdoor mesocosms at three application times. Means are followed by 84% confidence intervals; non-overlapping confidence intervals are significantly different ($\alpha = 0.05$).

Species	Application	LC10	LC50	LC90
Wood frog	Early (day 0)	1.45 (1.29, 1.57)	2.10 (2.00, 2.19)	3.03 (2.83, 3.34)
	Midway (day 7)	1.56 (1.07, 1.84)	2.44 (2.15, 2.79)	3.80 (3.20, 5.70)
	Late (day 14)	2.02 (1.47, 2.34)	4.27 (3.47, 7.42)	9.03 (5.83, 32.9)
American toad	Early (day 0)	0.99 (0.42, 1.35)	2.31 (1.86, 3.06)	5.36 (3.74, 15.2)
	Midway (day 7)	1.67 (0.72, 2.01)	2.30 (1.84, 2.89)	3.18 (2.63, 7.39)
	Late (day 14)	1.98 (1.49, 2.28)	3.93 (3.33, 5.83)	7.81 (5.44, 20.3)

Individual tadpole mass exhibited fewer treatment differences than tadpole survival. For wood frogs, mass was only reduced when exposed to late applications of 2 or 3 mg a.e./L. For American toads, mass was reduced when exposed to midway or late applications of 2 or 3 mg a.e./L.

Thus, the herbicide's effect on tadpole growth varied with application time and concentration, tending to have larger effects later in the experiment. The researchers also compared the effects of three smaller applications to one larger application that was added early, midway, or late in the experiment. In comparing a single application of 1 mg a.e./L to three applications of 0.33 mg a.e./L, there were no differences in survival or mass of the two anuran species. However, in comparing a single application of 3 mg a.e./L to three applications of 1 mg a.e./L, the single application caused lower survival of both species, regardless of when the single larger application was applied (although the late application of 3 mg a.e./L was not significant; $p = 0.088$). In terms of individual mass, both species were smaller when exposed to the single, larger application, but only when it was applied late in the experiment.

In the subset of nine treatments (three concentrations at three application times), the MANOVA found significant effects of glyphosate concentration, application time, and their interaction. Subsequent univariate analyses indicated that the main effects and their interactions were significant (or marginally non-significant; $p = 0.055$) for all four response variables. Mean comparisons indicated that, for wood frogs, increasing the concentration from 1 to 3 mg a.e./L caused a 91% decline in survival when applied early ($p < 0.0001$) and a 82% decline in survival when applied midway ($p < 0.0001$), but only a 27% decline in survival when applied late in the experiment ($p = 0.0008$). For American toads, increasing the concentration from 1 to 3 mg a.e./L caused a 75% decline in survival when applied early ($p < 0.0001$) and an 91% decline in survival when applied midway ($p < 0.0001$), but only a 31% decline in survival when applied late in the experiment ($p = 0.059$). Individual tadpole mass also showed interactive effects of concentration and application time that were either significant or marginally non-significant. For wood frogs, mean comparisons indicated that increasing the concentration of glyphosate from 1 to 3mg a.e./L caused no effect early in ontogeny ($p = 1.00$) or midway in ontogeny ($p = 0.99$), but a 27% decline in mass late in ontogeny ($p = 0.024$). For American toads, increasing the concentration of glyphosate from 1 to 3 mg a.e./L caused no change in mass early in the ontogeny ($p = 0.99$), but cased a 16% decline in mass midway in ontogeny ($p = 0.037$) and a 35% decline in mass late in ontogeny ($p < 0.0001$).

Table 2 (from Jones D. K. *et al.* 2010, ET&C, Vol. 29, No. 9, pp. 2016-25). Results of a multivariate analysis of variance examining the effects of glyphosate concentration and time of application on tadpole mass and survival.

Multivariate test	*df*	*F* value	*p* value
Application timing	8,42	9.3	<0.0001
Glyphosate concentration	8,42	21.1	<0.0001
Timing · Concentration	16,65	4.7	<0.0001

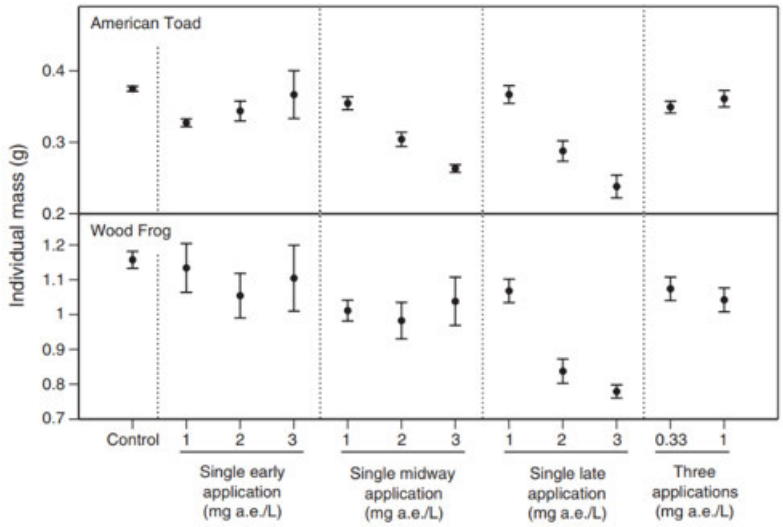


Figure 2 (from Jones D. K. *et al.* 2010, ET&C, Vol. 29, No. 9, pp. 2016-25). Mean individual mass of American toad and wood frog tadpoles exposed to varying Roundup Original MAX® concentrations (mg a.e. of glyphposate/L) at different times (day 0, 7, or 14). Data points represent mean individual mass (1 standard error) among the four replicates. Individual mass was recorded on day 18 following experimental takedown.

Periphyton and water quality

The analysis of periphyton mass exhibited an effect of time ($F_{1,36}$ = 21.4, $p < 0.001$), but no treatment ($F_{11,36}$ = 1.5, p = 0.19) or time-by-treatment interaction ($F_{11,36}$ = 1.1, p = 0.37).

The biomass of periphyton increased over time across all treatments. The analysis of dissolved oxygen exhibited an effect of treatment ($F_{11,36}$ = 4.6, $p < 0.001$), time ($F_{1,36}$ = 9.8, p = 0.004), and their interaction ($F_{11,36}$ = 2.6, p = 0.016). On both day 7 and day 14, separate ANOVAs detected significant treatment effects ($F_{11,36}$ = 2.1, p = 0.048; $F_{11,36}$ = 4.4, p = 0.003, respectively). On day 7, none of the treatments differed from the control (all $p > 0.05$), but the early application of 3 mg a.e./L caused 23% higher oxygen than the early application of 1 mg a.e./L ($p < 0.002$). On day 14, midway applications of 2 and 3 mg a.e./L had higher oxygen concentrations than the control ($p < 0.001$). In addition, early applications of 3 mg a.e./L continued to have higher oxygen concentrations than the early application of 1 mg a.e./L (p = 0.003).

The analysis of temperature exhibited an effect of treatment ($F_{11,36}$ = 2.3, p = 0.03), depth ($F_{1,36}$ = 3547, $p < 0.0001$) and time ($F_{1,36}$ = 3745, $p < 0.0001$). Among the possible interactions, there was no depth-by-treatment interaction ($F_{11,36}$ = 1.7, p = 0.12), but there were time-by-treatment ($F_{11,36}$ = 3.1, p = 0.005), time-by-depth ($F_{1,36}$ = 175, $p < 0.0001$), and time-by-treatment-by-depth ($F_{11,36}$ = 3.5, $p < 0.002$) interactions. As a result, the researchers analyzed the data within each sample date. On day 7, there was a depth effect ($F_{1,36}$ = 2263, $p < 0.0001$), but no treatment effect ($F_{11,36}$ = 1.0, p = 0.47) or treatment-by-depth interaction ($F_{11,36}$ = 0.5, p = 0.87). At this time, the water near the bottom of the mesocosm was approximately 38 °C colder than the water near the top of the mesocosm. On day 14, there was a depth effect ($F_{1,36}$ = 3138, $p < 0.0001$), treatment effect ($F_{11,36}$ = 6.2, p = 0.0001), and a depth-by-treatment interaction ($F_{11,36}$ = 3.4, p = 0.002). The interaction occurred because temperatures near the bottom of the mesocosm exhibited weaker treatment effects ($F_{11,36}$ = 2.3, p = 0.027) than temperatures near the top ($F_{11,36}$ = 7.5, $p < 0.0001$). Temperature differences among treatments at a particular depth were typically less than 18 °C whereas the difference between top and bottom temperatures was approximately 48 °C. Collectively, the data indicate that the mesocosms were colder near the bottom than the top.

The analysis of pH showed no effect of treatment ($F_{11,36}$ = 1.5, p = 0.19), but an effect of depth ($F_{1,36}$ = 130, $p < 0.0001$) and time ($F_{11,36}$ = 19, $p < 0.0001$). There also was a time-by-treatment interaction ($F_{11,36}$ = 2.4, p = 0.02), but no treatment-by-depth ($F_{11,36}$ = 1.3, p = 0.27), time-by-depth ($F_{1,36}$ = 0.2, p = 0.65), or time-by-depth-by treatment ($F_{11,36}$ = 1.5, p = 0.17) interactions. Because of these interactions, the researchers examined the data within each sample time. On day 7, pH showed a marginal effect of treatment ($F_{11,36}$ = 2.0, p = 0.055), an effect of depth ($F_{1,36}$ = 137, $p<0.0001$), and a depth-by-treatment

interaction ($F_{11,36}$ = 2.8, p = 0.009). On day 14, there was an effect of depth ($F_{1,36}$ = 70, p<0.0001), but no effect of treatment ($F_{11,36}$ = 1.8, p = 0.1) or a depth-by-treatment interaction ($F_{11,36}$ = 0.7, p = 0.76). On both day 7 and 14, pH was slightly higher near the top of the mesocosm (a difference of 0.08 pH units). Thus, differences in pH between the top and bottom of the mesocosm were small.

Conclusion

The present study demonstrated that the herbicide Roundup Original MAX®, composed of glyphosate and a surfactant, caused a range of mortality and growth effects that were dependent upon both the concentration and the timing of the application. The researcher pointed out that there is a need of many more studies of pesticide applications over ontogeny to determine whether these effects are due to differences in exposure duration or differences in sensitivity over ontogeny. The researcher also observed that multiple, sequential applications over time that accumulated to lethal concentrations were less lethal than one might expect based on concentration alone, opening the possibility that animals might be able to acclimate to the herbicide, although the underlying mechanism remains unclear. The present study also highlighted the ability of the herbicide to stratify in the water column, a phenomenon that has received very little attention, yet has important implications regarding both pesticide sampling on water bodies as well as how the habitat choices of individual organisms might make them more or less susceptible depending upon whether they move to strata that contain higher or lower pesticide concentrations. The implications of stratification are clear, but appear to be currently unexplored.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). Due to the test materials not being the representative formulation for the glyphosate EU renewal, the study is not relevant for the renewal. Roundup Original MAX® contains 48.7% glyphosate active ingredient.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning regarding non-relevance of the study. There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

This study demonstrates negative effects of Roundup Original MAX®, on amphibian growth (NOEC=1 mg a.e./L for both species) and survival (LC_{50} ranging 2.10-4.27 mg/L, depending on application time).

The RMS considers this study to be reliable.

Report author	Krynak K. L. *et al.*
Report year	2017
Report title	RodeoTM Herbicide Negatively Affects Blanchard's Cricket Frogs (*Acris blanchardi*) Survival and Alters the Skin- Associated Bacterial Community
Document source	Journal of Herpetology (2017), Vol. 51, No. 3, pp. 402-410
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

Utilizing the Blanchard's Cricket Frog (*Acris blanchardi*), a declining North American amphibian species, as the model, the researchers manipulated Rodeo^{TM} aquatic herbicide concentration and the life stage at which Rodeo exposure occurred. They assessed juvenile survival, time to metamorphosis, juvenile mass, and skin associated immune defense traits and found a 37% decrease in survival of larvae exposed to 2.5 mg a.e./L (acid equivalent) compared to controls despite that this commercial herbicide formulation does not contain an added surfactant. Surviving larvae exposed to 2.5 mg a.e./L Rodeo had structurally different larval skin bacterial communities compared to controls. Larval Rodeo exposure did not carry over to post-metamorphic traits (juvenile mass, juvenile skin bacterial community, juvenile natural peptide secretions). Rodeo treatments did not affect time to metamorphosis or juvenile survival. Rodeo concentration had marginally significant effects on juvenile mass and the juvenile skin bacterial community.

Materials and methods

The researchers obtained larvae from 12 *A. blanchardi* pairs collected from a single pond in Wood County, Ohio, USA. The researchers collected adult males and females and haphazardly paired frogs in 1-gal buckets containing pond water and plastic aquarium plants. Each pair produced 20-100 eggs. Larvae hatched 15-22 June 2013. The researchers randomly assigned larvae to treatments on 27 June 2013. The researchers used four exposure concentrations (control: no Rodeo; and low, medium, and high Rodeo; see details below) and three exposure stages (larval exposure, post-metamorphic juvenile exposure, or exposed as both larvae and juveniles) for a total of 10 treatments. The researchers established five replicates of three larvae each per treatment (i.e., the experimental unit is the replicate), but replicate number was reduced by high mortality following high Rodeo larval exposure. To account for this and maximize the ability to detect carryover effects of high Rodeo exposure, those replicates originally assigned as high Rodeo juvenile became only control replicates, and those replicates originally assigned as high Rodeo larval + juvenile became high Rodeo larval only (i.e., not exposed as juveniles, only exposed as larvae). The researchers conducted the experiment in an indoor animal facility at Case Western Reserve University maintained at 25.5-27.78° C with a 12 h: 12 h light dark cycle. Each replicate (three larvae) was a 15-L Sterilitee (Massillon, Ohio, USA) tank filled with 10 L of dechlorinated water with plastic aquarium plants provided for cover (50 tanks in total). The researchers conducted 50% water/treatment solution changes every other day for the duration of the larval period via static renewal (Relyea, 2004). The researchers fed larvae ad libitum TetraMine (Blacksburg, Virginia, USA) sinking tropical tablets daily (0.08 g per tank) and siphoned all uneaten food and solid waste daily. Upon metamorphosis (stage 42; Gosner, 1960), after swabbing for microbial communities (detailed below). The researchers moved these juveniles to ventilated 1-L plastic cups containing 100 mL of dechlorinated water/treatment solution, and plastic aquarium plants. The researchers raised juveniles from the same replicate together, so juvenile group size was 1-3, dependent on larval survival.

The researchers performed 100% water changes every other day for juveniles, and fed them ad libitum daily with *Drosophila melanogaster* dusted with RepCale (Los Gatos, California, USA) vitamin supplement. Rodeo treatments included four exposure concentrations (based on milligrams per liter of the acid equivalent, glyphosate) reflecting glyphosate concentrations documented in nature (Feng *et al.*, 1990; Thompson *et al.*, 2004; Relyea, 2005) and below the maximum concentrations expected when spraying emergent aquatic vegetation (>3.7 mg a.i./L; Giesy *et al.*, 2000).

The researchers report glyphosate concentration as both acid equivalent (a.e.) and active ingredient (a.i.) for comparison with extant literature: control, 0.0 mg a.e./L (0.0 mg a.i./L); low, 0.75 mg a.e./L (1.01 mg a.i./L); medium: 1.5 mg a.e./L (2.02 mg a.i./L); and high, 2.5 mg a.e./L (3.38 mg a.i./L). The researchers conducted exposures for 12 d, a conservative approach, because glyphosate has a half-life of 12-70 d, depending on the microbial characteristics of the habitat (U.S. Environmental Protection Agency, 1992; Zaranyika and Nyandoro, 1993). The researchers began exposures 1) 6 d after larvae were randomly assigned to replicates (larval; larvae 11–18 d old), 2) 10 d after the final larvae in a replicate reached metamorphosis (juvenile), or 3) during both developmental stages. Because of logistical constraints, experiment end-day varied by up to 6 d. To effect the Rodeo manipulation, the researchers added Rodeo commercially formulated product (53.8% glyphosate, confirmed by Mississippi State Chemical Laboratory) to appropriate tanks on Day 1, bringing concentration to 50% of treatment concentration (8 µL, 16 µL, or 26 µL of Rodeoe formulated product for low, medium, and high exposures, respectively); the researchers mixed all tanks, including controls, thoroughly. On Day 2, the researchers repeated this process, bringing Rodeo concentrations to prescribed treatment levels: control, low, medium, and high. Beginning on Day 4, the researchers conducted 50% (5 L) water changes via static renewal every other day (Relyea, 2004). On Day 4 (5 July 2013) a mistake was made during the water change that resulted in Rodeo concentrations being elevated temporarily (low, 1.125 mg a.e./L; medium; 2.25 mg a.e./L, high, 3.75 mg a.e./L); this error was caught the next day and remedied with water changes that brought concentrations to the intended levels.

This elevated glyphosate concentration (highest = 3.75 mg a.e./L) is still below the highest concentration expected when spraying aquatic vegetation (Giesy *et al.*, 2000; Relyea, 2005). On Day 15, prior to the water change, the researchers tested pH and ammonia levels in all larval tanks. Rodeo addition significantly decreased water pH, but this small difference is unlikely to be biologically important (mean ± SE: control = 7.61 ± 0.01, low = 7.54 ± 0.01, medium = 7.52 ± 0.02, high = 7.51 ± 0.01; $F_{3,46} = 14.15$, $P < 0.0001$; Pierce, 1985). Ammonia levels were significantly higher in medium and high Rodeo larval tanks compared to controls (control, 0.34 ± 0.03 mg/L; low, 0.40 ± 0.04 mg/L, Z = -1.35, P = 0.18; medium, 0.50 ± 0.00 mg/L, Z= -3.5073, P = 0.0004; high, 0.50 ± 0.00 mg/L, Z= -3.5073, P = 0.0004 Kruskal-Wallis test; for three comparisons, the Bonferroni corrected $\alpha = 0.017$), but again these levels are unlikely to negatively affect larval survival (Jofre and Karasov, 1999). Juvenile treatments were conducted with identical concentrations also for 12 d. The researchers conducted 100% water changes on juvenile cups every other day (100 ml/cup). Juveniles had the opportunity to climb out of direct contact with the treatment solutions, as would be the case in the natural environment. The researchers collected skin-associated bacterial community samples from each metamorphosing larvae during the transfer to juvenile housing and collected juvenile bacterial community samples immediately prior to collection of natural peptide secretions at the experiment's end (22–28 d after the last larvae metamorphosed in a replicate). The researchers collected bacterial community samples (skin swabs) and natural peptide secretions following Krynak *et al.* (2015). The unit of analysis was the replicate (1-3 individuals): natural peptide secretions were collected simultaneously from all individuals in the replicate, and the researchers collected mass on all individuals subsequent to euthanasia (MS-222) that immediately followed peptide collection. The researchers collected the mass data with an analytical scale after blotting the MS-222 solution from the carcasses of the frogs. The researchers averaged time to metamorphosis (days from assignment to replicate to reaching Gosner stage 42) and mass by replicate.

The researchers extracted bacterial DNA from skin swabs, pooling swabs by tank, using a bead beating and phenol chloroform extraction method (Burke *et al.*, 2006, 2008). The researchers amplified bacterial DNA with the use of 16S rRNA gene primers: 338f and 926r, following Carrino-Kyker *et al.* (2012). With the use of terminal restriction fragment length polymorphism profiling (TRFLP), the researchers examined bacterial community structure across treatments (Krynak *et al.*, 2015, 2016). TRFLP profiles

were processed using the TRFLPR package (Petersen *et al.*, 2015; R Core Team, 2013). The researchers used nonmetric multidimensional scaling analyses (NMDS) to assess bacterial community structure across treatments in PC-ORD (Version 5.0; Bruce McCune and MJM Software, 1999). The researchers used axis scores from the resulting NMDS ordination solution to assess influence of treatments on the variation across each NMDS axis independently to maximize statistical power (see statistical analysis description below). Differences in NMDS axis scores indicate differences in the taxonomic composition of the bacterial community on the amphibians' skin. The researchers processed natural peptide secretion samples as in Krynak *et al.* (2015), and utilized a Micro BCA Protein Assay Kit (product 23235; Rockford, Illinois, USA) for analysis of total protein concentration, standardizing for total frog biomass per tank (Krynak *et al.*, 2015). The researchers measured natural peptide secretion bioactivity by determining pathogen growth rate in culture when challenged by natural peptide secretions from frogs following Krynak *et al.* (2015). The researchers read optical density (OD; BioRad Imark, Hercules, California, USA) at 490 nm on Day 0 (immediately after plating), Day 1 (13 h postplating), Days 2-9. The researchers fit a logistic growth model to data using a self-starting nls logistic model function (R Development Core version 3.0.2, 'stats' package, Jose´ Pinheiro and Douglas Bates), and Bd growth rate (r) was determined (Krynak *et al.*, 2015). The researchers used Bd growth rate (r) as the proxy for bioactivity of the natural peptide secretions; rapid growth rate indicated natural peptide secretions with reduced bioactivity against Bd.

Statistical Analysis

The researchers tested if the treatments affected larval and juvenile percent survival per tank utilizing a Kruskal-Wallis test for multiple comparisons. The researchers compared survival in each treatment to survival in the control group. To account for multiple comparisons, the researchers applied Bonferroni correction (Bonferroni corrected $\alpha = 0.05/n$, n = number of comparisons). The researchers used analysis of variance (ANOVA) to test if Rodeo treatments applied during the larval stage affected larval duration or any of the three axes of the NMDS ordination of the larval bacterial community. In these models, each response variable was analyzed with a single predictor variable (larval Rodeo concentration) with four levels (control, low, medium, and high) via ANOVA. The researchers included replicates that underwent post-metamorphic (juvenile) treatments in these analyses of larval traits; replicates that received juvenile-only Rodeo exposures were incorporated into the control group, and replicates that received both larval and juvenile Rodeo exposures were incorporated into the larval groups. The researchers also tested whether larval-stage Rodeo exposure (four levels: control, low, medium, and high) carried over to affect post-metamorphic juvenile traits (average juvenile mass, log-transformed natural peptide secretion production, log-transformed bioactivity of the natural peptide secretions, and each of the three NMDS ordination axes describing juvenile bacterial community structure) using analysis of covariance (ANCOVA). Average age (in days) post-metamorphosis was included as a covariate in each model to account for the possible confounding factor of age at time of juvenile sampling. Finally, the researchers used ANCOVA to test if Rodeo treatments affected post-metamorphic juvenile traits, including average age post-metamorphosis as a covariate in each model. In these ANCOVA models the researchers assessed each of the responses (average juvenile mass, log-transformed natural peptide secretion production, log-transformed bioactivity of the natural peptide secretions, and each of the three NMDS ordination axes describing juvenile bacterial community structure) as a function of the stage at which the animals were exposed to Rodeo (three levels: larval exposure, juvenile exposure, or both larval and juvenile exposure) and the concentration of Rodeo to which they were exposed (two levels: low or medium). Control and high Rodeo concentration treatments were excluded in these particular analyses to create a balanced design and meet the assumptions of the model. The researchers included age post-metamorphosis as a covariate in each model. Interactions were not included because of low statistical power associated with small sample size. The researchers utilized Type III sums of squares for all ANOVA/ANCOVA analyses. Planned contrasts were used to compare treatment means in all ANOVA/ANCOVA models.

Results

Larval Traits

Survival to metamorphosis in the high Rodeo treatment was 37% lower than in controls (Z = 2.688, P = 0.007; for three comparisons, the Bonferroni corrected α = 0.017), but larval survival did not differ between control and low (Z = 0.284, P = 0.776) or medium (Z = -0.174, P = 0.862) Rodeo treatments.

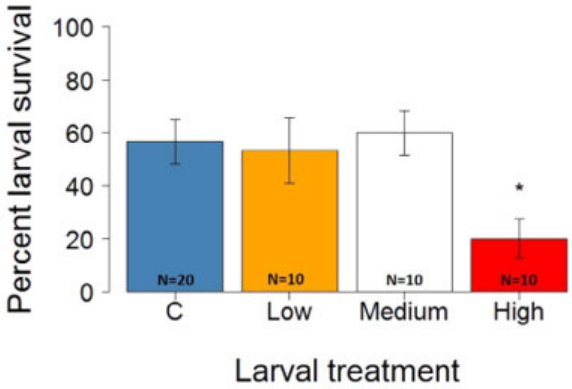


Figure 1 (from Krynak K. L. *et al.* 2017, J. Herpetol, Vol. 51, No. 3, pp. 402-410). Larval *Acris blanchardi* survival in response to Rodeo™ (Dow AgroSciences, LLC) concentration. High Rodeo concentration for a period of 12 d reduced survival by 36.67% compared to control (Kruskal-Wallis test with Bonferroni correction for multiple comparisons; Z = 2.69, P = 0.0017). N = number of replicates at beginning of the experiment.

The larval bacterial community was marginally affected by larval Rodeo concentrations along NMDS Axis 2 (Axis 1: $F_{3,33}$ = 1.63, P = 0.20; Axis 2: $F_{3,33}$ = 2.63, P = 0.07; Axis 3: $F_{3,33}$ = 0.41, P = 0.75). Post hoc planned contrasts indicated a significant difference in the larval bacterial community between high Rodeo (2.5 mg a.e./L) and control (Axis 2: T = 2.8, P = 0.009) treatments, but there were no differences between low and medium Rodeo treatments and controls. Time to metamorphosis was not affected by exposure to Rodeo (77.29 6 2.27 d; Rodeo concentration: $F_{3,33}$ = 0.16, P = 0.92).

Post-metamorphic (Juvenile) Traits

Survival from metamorphosis to the end of the experiment did not significantly differ between control and any Rodeo treatment (low larval exposure: Z = 0.053, P = 0.958; low juvenile exposure: Z = -0.472, P = 0.637; low larval + juvenile exposure : Z = -0.479, P = 0.632; medium larval exposure: Z = 0.217, P = 0.828; medium juvenile exposure: Z = 0.840, P = 0.401; medium larval and juvenile exposure: Z = -1.461, P = 0.1440; high larval exposure: Z =-1.461, P = 0.144; for seven comparisons, the Bonferroni corrected α = 0.007).

The researchers found no evidence of carryover effects of larval Rodeo on juvenile mass, natural peptide secretion production, bioactivity of the natural peptide secretions, or any of the juvenile bacterial community NMDS ordination axes in ANCOVA models. When examining possible additive effects of treatments, the researchers found a marginal effect of Rodeo concentration on juvenile mass; however, if controlling for multiple comparisons, the effect was not significant. Juveniles were larger in the medium Rodeo treatment than in the low treatment (low: 0.30 ± 0.02 g; medium: 0.38 ± 0.02 g; $F_{1,19}$ = 4.43, P = 0.05). The researchers did not find significant effects of Rodeo concentration or the timing of Rodeo exposure on natural peptide secretion production or bioactivity (natural peptide secretion production: 252.84 ± 30.24 lg/mL per gram body weight; bioactivity: 1.00 ± 0.05). The researchers did not find evidence of a strong effect of Rodeo concentration or life stage of exposure on the juvenile bacterial community, but the researchers did find a marginal effect of Rodeo concentration on the juvenile bacterial community along NMDS Axis 3 (Axis 3: $F_{1,19}$ = 4.24, P = 0.06).

Conclusion

The researchers conclude that Rodeo exposure at field-relevant concentrations can increase mortality in *A. blanchardi*, a species already suffering population declines and extirpations in the northern portions of its range (Gray and Brown, 2005; Lehtinen and Skinner, 2006; Gamble *et al.*, 2008).

Additionally, the researchers showed that Rodeo could change the skin associated bacterial community structure, which may indirectly decrease amphibian fitness. Improving the knowledge of the influence herbicide use has on amphibians across life stages provides an opportunity for changes to application strategies to protect amphibian health or, at minimum, lessen negative effects of the practice.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). Due to the test materials not being the representative formulation for the glyphosate EU renewal, the study is not relevant for the renewal.

Rodeo™ Original MAX®, commercialized by Dow AgroSciences LLC, contains 53.8% glyphosate and unknown surfactant system.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning regarding the non-relevance of the study. There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The fact that all test larvae originate from adults collected from the same pond means that any potential background exposure to glyphosate (or other stressors) is also accounted for by the control organisms.
The study demonstrated increased mortality and changes in skin microbiota of amphibian larvae (*Acris blanchardi*) exposed to environmentally-relevant concentrations of Rodeo™ Original MAX®. The NOEC for both parameters is 2.02 mg a.i./L (corresponding to 1.5 mg a.e./L). No effect on juvenile survival or skin microbiota was observed, thus NOEC is 3.38 mg a.i./L (corresponding to 2.5 mg a.e./L).

The RMS notes that pH decreased and ammonia increased in high Rodeo™ Original MAX® test solutions, but agree with the authors' reasoning that these changes do not have a biological impact, as they are still within the normal range of the test organisms.

The RMS considers this study to be reliable with restrictions, since no analytical confirmation of test concentrations was carried out.

Data point CA 9

Report author	Lanctot C. *et al.*
Report year	2014
Report title	Effects of glyphosate-based herbicides on survival, development, growth and sex ratios of wood frog (*Lithobates sylvaticus*) tadpoles. II: agriculturally relevant exposures to Roundup WeatherMax® and Vision® under laboratory conditions.
Document source	Aquatic toxicology (2014), Vol. 154, pp. 291-303
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The purpose of this study was (1) to determine if an agriculturally relevant exposure to Roundup WeatherMax® influences the development of wood frog tadpoles (*Lithobates sylvaticus*) through effects on the mRNA levels of genes involved in the control of metamorphosis; (2) to compare results to the well-studied Vision® formulation (containing the isopropylamine salt of glyphosate [IPA] and polyethoxylated tallowamine [POEA] surfactant) and to determine which ingredient(s) in the formulations are responsible for potential effects on development; and (3) to compare results to recent field studies that used a similar experimental design. In the present laboratory study, wood frog tadpoles were exposed to an agriculturally relevant application (i.e., two pulses) of Roundup WeatherMax® and Vision® herbicides as well as the active ingredient (IPA) and the POEA surfactant of Vision®. Survival, development, growth, sex ratios and mRNA levels of genes involved in tadpole metamorphosis were measured. Results show that Roundup WeatherMax® (2.89 mg acid equivalent (a.e.)/L) caused 100% mortality after the first pulse. Tadpoles treated with a lower concentration of Roundup WeatherMax® (0.21 mg a.e./L) as well as Vision® (2.89 mg a.e./L), IPA and POEA had an increased condition factor (based on length and weight measures in the tadpoles) relative to controls at Gosner stage (Gs) 36/38. At Gs42, tadpoles treated with IPA and POEA had a decreased condition factor. Also, at Gs42, the effect on condition factor was dependent on the sex of tadpoles and significant treatment effects were only detected in males. In most cases, treatment reduced the normal mRNA increase of key genes controlling development in tadpoles between Gs37 and Gs42, such as genes encoding thyroid hormone receptor beta in brain, glucocorticoid receptor in tail and deiodinase enzyme in brain and tail.

Materials and methods

Tadpoles (n = 50) were placed into 50-liter glass aquaria (1 tadpole/L) and divided into seven treatment groups (3 replicates per treatment): control, Roundup WeatherMax® (0.21 and 2.89 mg a.e./L), Vision® (2.89 mg a.e./L), isopropylamine (IPA) salt of glyphosate (2.89 mg a.e./L), polyethoxylated tallowamine (POEA, 1.43 mg/L, pulse exposure) and POEA chronic (1.43 mg/L, continuous exposure until tadpoles reached Gs42). Tadpoles were fed a combination of boiled kale and Ward's dry tadpole food daily ad libitum, supplemented with algal pellets weekly.

Treatments were added to experimental aquaria twice except for POEA chronic. The first application took place when tadpoles reached Gs25, which corresponds approximately to the developmental stage that *L. sylvaticus* tadpoles would reach when the first pulse application would be applied for agriculture. The second application took place 2 weeks later (~Gs30). Each pulse exposure was carried out over 4 consecutive days with full water renewal each day. During each 4-day pulse, nominal concentrations were decreased by 25% each day to mimic natural degradation of the herbicide. After the fourth day, tadpoles were transferred to and remained in clean water until they reached Gs42. Water renewals were

performed three times per week between and after each pulse exposure. The POEA chronic treatment was maintained at a target concentration of 1.43 mg/L POEA until tadpoles reached Gs42 and POEA treatment was renewed at the same time as the pulse treatments. Water samples (1 L) were taken on each day of the 4-day exposure and additionally on day 5 for each pulse to determine glyphosate concentrations. Samples were maintained at ~6 °C and analyzed within 11 days. Aqueous glyphosate concentrations were measured using a liquid chromatography/mass spectrometry method. Temperature, dissolved oxygen and pH measurements were recorded regularly (approximately 3-5 times per week). Survival was assessed regularly throughout the experiment.

Tadpoles were sampled for analysis of mRNA levels when the median developmental stage within each aquarium reached Gs31 (pre-metamorphosis), Gs37 (pro-metamorphosis) and Gs42 (metamorphic climax). For each developmental stage, tadpoles were sampled and morphometrics were measured from one aquarium per treatment (i.e., Gs31, Gs37, Gs42 were sampled from different aquaria). Subsequently, tadpoles were dissected and whole brain and tail tissues were preserved in RNAlater (Ambion), a reagent used to stabilize RNA.

Five males and three females per treatment were randomly selected for histological assessment to determine presence of testicular oocytes, inhibited gonadal development, oocyte atresia or other potential abnormalities. Mesonephrosgonad complexes were embedded in paraffin blocks and oriented in transverse plane for sectioning. Paraffin sections were cut at 6 µm and stained with haematoxylineosin following conventional histological procedures.

Total RNA from whole brain and tail tissue from Gs37 and Gs42 tadpoles was isolated using Qiagen RNeasy Micro Kit, or a combined protocol of TRIzol (Invitrogen) and Qiagen RNeasy Mini Kit, respectively. The concentrations of total RNA, the ratio of absorbance at 260 and 280 nm and the ratio of absorbance at 260 and 230 nm were determined using a spectrophotometer. Total cDNA of brain and tail samples was synthesized from 1 and 3 µg of total RNA, respectively.

Real-time RT-PCR was performed using SYBR Green I for trβ, grII, crf and rpl8, and fluorogenic 51 nuclease chemistry for dio2 and dio3. NORMA-Gene, a data driven normalization algorithm, was used to normalize mRNA level data. Normalization was performed using 6 target genes analyzed for each tissue. Fold change in normalized mRNA relative to control Gs37 was then calculated for each sample. Biological replicates were averaged to obtain mean fold change in mRNA levels ± standard error of the mean (SEM).

Data analysis
Differences in growth measurements (SVL, tail length, weight and condition factor) between treatments were analyzed for significance using non-parametric Kruskal-Wallis test with pairwise multiple comparisons. At Gs42 analysis for alterations in sex ratios of the treated groups relative to the control and relative to an equal proportion of males and females was performed using the Chi-Square test. In addition, differences between sexes in SVL, tail length, weight, condition factor (k) and time to metamorphosis were analyzed by Mann-Whitney U test. Levels of mRNA were analyzed for normality (Kolmogorov-Smirnov) and homogeneity of variance (Levene's test) and log10 transformed to meet parametric assumptions. Two-way ANOVA was performed with treatment and developmental Gosner stage as independent factors and mRNA levels as the dependent variables. Tukey's HSD post hoc multiple comparisons were used to analyze for statistical differences between treatments and developmental stages. Analyses were performed using SPSS 20.0.0 (IBM Corp.) and differences were considered to be significant when $p < 0.05$.

Results

Growth, survival and development
16 days after the first exposure to Roundup WeatherMax® (2.89 mg a.e./L) survival was 5.3% and complete mortality was observed before the second pulse (day 18). Similarly, in the chronic POEA (1.43 mg/L) treatment, survival was 11.3% at 16 days after the beginning of the exposure (approximately 8 times lower than control), and by day 23, all test specimens died. By the end of the experiment (after 2

pulses), survival was 78% in control, 86% in Roundup WeatherMax® (0.21 mg a.e./L), 68% in Vision® (2.89 mg a.e./L), 86% in IPA (2.89 mg a.e./L) and 70% in POEA pulse (1.43 mg/L).

At Gs31, no significant differences in SVLm tail length or condition of tadpoles were observed. Tadpoles exposed to one pulse of Roundup WeatherMax® (0.21 mg a.e./L), Vision® (2.89 mg a.e./L), IPA (2.89 mg a.e./L) and POEA (1.43 mg/L) weighed 7.9, 7.1, 12.9 and 6.4% more than control tadpoles, respectively. Tadpoles exposed to two pulses of Roundup WeatherMax® (0.21 mg a.e./L), Vision® (2.89 mg a.e./L), IPA (2.89 mg a.e./L), and POEA (1.43 mg/L) had 11.3, 5.8, 8.4, 5.8% smaller SVL than control tadpoles, respectively. Tadpoles exposed to two pulses of POEA (1.43 mg/L) also had a 7.4% increase in tail length relative to control. No significant differences in tail length were observed in the Roundup WeatherMax® (0.21 mg a.e./L), Vision® (2.89 mg a.e./L) and IPA (2.89 mg a.e./L) treatments relative to controls. In addition, tadpoles exposed to Roundup WeatherMax® (0.21 mg a.e./L), Vision® (2.89 mg a.e./L), IPA (2.89 mg a.e./L), and POEA (1.43 mg/L) had 32.3, 18.8, 34.7, 22.6% higher condition factor than control, respectively. At metamorphic climax (Gs42), no significant differences in SVL, weight or tail length were observed between treatments. Tadpoles exposed to IPA (2.89 mg a.e./L) and POEA (1.43 mg/L) had 28.0 and 22.2% lower condition factor than controls, respectively. There were no treatment related differences in time to metamorphosis.

Sex ratios and gonadal morphology
There were no differences for sex ratios between control and treatment groups except for the IPA (2.89 mg a.e./L) treatment, with an increase in the proportion of females to 55.2%, compared to 41.1% in the control group. Intersex (presence of both ovarian and testicular tissue in the same individual) was not observed in this study. In addition, there were no anomalies observed in the gonads.

mRNA levels in brain and tail
The effect of treatment in brain was significant for crf, trβ, and dio3 but was not significant for grII. Tadpoles exposed to IPA (2.89 mg a.e./L) had significantly less tr mRNA than all other treatments. Although two-way ANOVA showed significant differences for crf and dio3, Tukey post hoc tests did not detect significant differences between treatments. Significant interactions between Gosner stage and treatment were detected for dio2 and rpl8 in the brain. rpl8 mRNA levels were significantly increased by Roundup WeatherMax® (0.21 mg a.e./L) and reduced by POEA (1.43 mg/L) treatment at Gs42. No interactions were detected for all other genes (trβ, dio3, crf and grII).

In tail tissue, a two-way ANOVA analysis also revealed stage and treatment differences, and significant stage treatment interactions for some of the genes analyzed. Simple main effects analysis showed significant differences between Gosner developmental stage for crf, dio2, and dio3. Specifically, an increase in dio2 and dio3 mRNA levels and a decrease in crf mRNA levels between Gs37 and Gs42 was observed. Tadpoles exposed to IPA (2.89) had significantly less dio2 mRNA than did tadpoles exposed to Roundup WeatherMax® (0.21 mg a.e./L) and POEA (1.43 mg/L) ($p < 0.05$), but they did not differ from control and Vision® (2.89 mg a.e./L) groups. Tadpoles exposed to IPA (2.89 mg a.e./L) also had significantly less dio3 mRNA than control and Vision® (2.89 mg a.e./L) but did not differ from Roundup WeatherMax® (0.21 mg a.e./L) and POEA (1.43 mg/L) ($p > 0.05$). Significant interactions between Gosner stage and treatment were detected for trβ, grII and rpl8. For these 3 genes, the normal developmental changes in mRNA levels between Gs37 and Gs42 observed in the control group were altered by treatment. At Gs42, trβ mRNA levels were reduced by Roundup WeatherMax® (0.21 mg a.e./L) and IPA (2.89 mg a.e./L) treatment. Similarly, grII mRNA levels were also reduced by Roundup WeatherMax® (0.21 mg a.e./L) treatment at Gs42. For rpl8, mRNA levels were reduced by both IPA (2.89 mg a.e./L) and POEA (1.43 mg/L) treatment at Gs42.

Conclusion

The current laboratory study shows that exposures of wood frog tadpoles to Roundup WeatherMax® alter the mRNA levels of genes involved in the control of metamorphosis. These effects were observed at an exposure concentration of 0.21 mg a.e./L applied at two 4-day pulses, but concentrations were not high enough to translate into clear phenotypic or developmental changes. Time to metamorphic climax (Gs42) was not affected. Furthermore, exposure to Roundup WeatherMax® and chronic exposure to

POEA (1.43 mg/L) is highly toxic to *L. sylvaticus* tadpoles under laboratory conditions. Survival, growth and mRNA results also indicate that Roundup WeatherMax® has greater toxicity than Vision® formulation containing the POEA surfactant. Furthermore, pulse exposure to POEA (1.43 mg/L) did not cause a greater disruption of growth, development, or mRNA abundance of thyroid-dependent genes than the active ingredient (IPA). In the discussion section, differences between laboratory and field studies are discussed. It is concluded that the effects of glyphosate-based herbicides are exaggerated in laboratory studies.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). None of the tested formulations is the representative formulation for the glyphosate EU renewal. In addition, the tested formulation Vision® contains POEA surfactant that is not permitted for use in formulated herbicidal products in the EU.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning regarding extrapolation of the results to EU relevant conditions, particularly as this is a laboratory study. The fact that all test organisms were collected from the same wetlands means that any potential background exposure to glyphosate (or other stressors) is also accounted for by the control organisms.

Regarding the formulation Vision®, the results are not considered relevant for the assessment of the representative product since the tested formulation includes surfactants that are similar to substances that are not allowed in the EU (Regulation (EU) 2016/1313 and/or Regulation (EU) 2021/383.

Regarding Roundup WeatherMax® formulation, there is not sufficient information in the study report to demonstrate whether it is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The effects caused by the Roundup WeatherMax® formulation include: 100 % mortality of tadpoles at day 18, after exposure to one pulse (96 h) of 2.89 mg a.e./L, increased weight (by 7.9%) and decreased SVL (by 11.3%) relative to control, after one, respectively two 0.21 mg a.e./L pulses. Thus, NOEC for survival is 0.21 mg a.e./L, whereas NOEC for body weight and SVL could not be determined, as the effects were significant at the lowest tested concentration.

The RMS notes that survival after 2 pulses was lower for the control organisms than for those exposed to Roundup WeatherMax® (78 vs 86 %).

The RMS considers this study to be reliable.

Report author	Munoz L. M. H. *et al.*
Report year	2015
Report title	Toxicity assesment of two agrochemicals, Roundup Active and Cosmo-Flux411F,21 to colombian anuran tadpoles Original title: Evaluación de la toxicidad de dos agroquímicos, Roundup® Activo y Cosmo-Flux®411F, en renacuajos de anuros colombianos
Document source	Acta Biologica Colombiana (2015), Vol. 20, No. 2, pp. 153-161
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The purpose of this study was to determine the median lethal concentration (LC_{50}) and some of the sub-lethal effects (changes in body size and swimming performance) caused by Roundup® Activo and an coadjuvant Cosmo-Flux®411F in the tadpoles of four species of Colombian anurans exposed under controlled laboratory conditions and in microcosm. The species that was most sensitive to Roundup® Activo was *Hypsiboas crepitans* (Laboratory: LC_{50} = 1414 µg a.e. of glyphosate/L and Microcosm: LC50 = 4 kg a.e. of glyphosate/ha) and for Cosmo-Flux®411F *Rhinella humboldti* (Laboratory: LC_{50} = 319 mg/L and Microcosm: LC_{50} = 632.3 L/ha). In both the laboratory and microcosm, exposure to Roundup® Activo did not alter the body size or swimming performance of the tadpoles, while Cosmo-Flux®411F altered body size but had no effect on swimming performance. When the LC_{50} of the two agrochemicals were compared to the concentrations used in the field, a moderate risk was associated with Roundup® Activo while Cosmo-Flux®411F proved not to be lethal. Furthermore, Roundup® Activo was significantly more toxic than Cosmo-Flux®411F.

Materials and methods

This study was conducted on growth stage 25 tadpoles (Gosner, 1960) using four species of Colombian anurans: *Rhinella marin*a (Linnaeus, 1758), *Rhinella humboldti* (Gallardo, 1965), *Hypsiboas crepitans* (Wied-Neuwied, 1824) and *Engystomops pustulosus* (Cope, 1864).

Egg batches were collected for these species from department of Tolima, Colombia and transported to the Herpetology laboratory at the University of Tolima in separate plastic containers that were filled with collection site water, and transfered to containers with dechlorinated aerated water, at temperature of 23-24°C, until they reached stage 25 (Gosner, 1960). The tadpoles were not fed before or during the experiments.

Assessed Agrochemical
Roundup® Activo: A potassium salt of glyphosate-based herbicide (363 g/L of glyphosate acid). Glyphosate concentrations for agricultural applications was 1.77 kg a.e./ha and 3.69 kg a.e./ha for controlling illicit crops in Colombia.

Cosmo-Flux®411F: is a stereo-specific, non-ionic co-adjuvant, whose active ingredient is a mixture of hexitan esters (17%) and liquid isoparaffins additives (83%). The product is licensed for sale (number 2186) by the Colombian Institute of Agriculture ICA (Cosmoagro, 2014) and the Colombian Ministry of Health has it listed as Toxicological Category IV (slightly toxic to human health). The use

concentrations suggested by Cosmoagro®, the company that markets Cosmo-Flux®411F, was 0.5 to 1 L/ha, or between 1.5 and 10 mL/L, equal to 255 and 1700 mg/L.

Experiments conducted in controlled laboratory conditions
for 96 hours, with 20 tadpoles from each species divided between two replicates, per five exposure concentrations of Roundup Activo at concentrations of 325; 750; 1500; 3000 and 6000 µg a.e. of glyphosate/L; and of the Cosmo-Flux411F co-adjuvant: 106.25; 212.5; 425; 850 and 1700 mg/L. Each duplicate had a negative control (dechlorinated water). Test vessel volume per replicate was 1000 mL in glass, 2 liter aquariums. The water used to prepare the solutions had been previously dechlorinated through aeration. The test was conducted using a semi-static test design, renewed every 24 hours, water quality, conductivity, dissolved oxygen, alkalinity, hardness, temperature and pH were measured before and after renewals. The light: dark photoperiod was 12:12 hours, maintained through white light lamps (Phillips TLT 20W/54RS) connected to a digital timer (General Electric PM621). The median lethal concentration (LC50) for each species was determined. These 96-hour LC50 values were calculated using the TSK Trimmed Spearman-Karber method (Version 1.5) (Hamilton *et al.*, 1977).

Experiments in microcosm conditions
Tadpoles of all species were also exposed in duplicate microcosms per treatment group. The microcosms were polyethylene containers measuring 70 cm diameter and 13 cm depth (experimental area = 0.1520 m^2), to which a layer of earth (450 g) and sand (647 g) was added, obtained from the botanical gardens at the University of Tolima The sediment was covered with a white (muslin) cloth, for easier subsequent observations and organism counts. Ten liters of the experimental solution was added, prepared with previously dechlorinated water, (2.5 tadpoles/liter), plus detritus (two dry leaves), stones (four to five small stones, 100 g) and a macrophyte. These microcosms were maintained in a laboratory at 24 ± 2°C and a light: dark photoperiod of 12: 12. Microcosm were left for at least 1 hour before organisms addition in the center of the containers.

Experimental duration was 96 hours, with 50 tadpoles in total (divided between two replicates) exposed to five concentrations of the Roundup Activo herbicide at the equivalent of 1.845; 3.69; 7.38; 14.76 and 29.52 kg a.e. of glyphosate/ha; and of the Cosmo-Flux411F coadjuvant: 205.5 (= 53.1 mg/L); 410.9 (= 106.2 mg/L); 821.9 (= 212.4 mg/L); 1643.9 (= 424.8 mg/L) and 3287.9 L/ha (= 849.6 mg/L), adjusted to the experimental area. Negative control replicates were also included (dechlorinated water only). The test was conducted static. Water quality, pH, temperature, conductivity and dissolved oxygen were recorded at 0, 24, 48, 72 and 96 hours and at an average depth. Alkalinity and hardness were recorded at start and end of the 96 hour exposure period. The mean and standard deviations for the physico-chemical parameters were recorded at 0 and 96 hours. LC_{50} values were determined as previously described for the laboratory study.

Assessment of the Sub-lethal Effects
Surviving tadpoles from the laboratory and microcosm experiments, where the survival rate was >50% for all species, were sampled to analyze for sub-lethal effects of the herbicide Roundup Activo and the coadjuvant Cosmo-Flux411F. Only tadpoles without apparent deformities were selected, with ten tadpoles sampled from the laboratory trials and twenty from the microcosm trial. All tadpoles were photographed to measure body width, body length, tail length and total length using the ImageJ program and analysis using a multivariate analysis of variance (MANOVA) and a factorial analysis of variance (ANOVA) to establish differences in the morphometric measurements. Swimming capacity was also assessed by stimulating the tadpole's tail with a brush to cause it to swim across a 50 cm long x 1 cm wide x 1 cm high track. Three replicate runs were conducted per tadpole, and the maximum distance travelled (cm) and maximum speed attained (cm/s) were recorded. This information was compared using a multivariate analysis of covariance (MANCOVA) and subsequent analyses of covariance (ANCOVA), using the total length of the organisms as a covariate.

Results

The species mortality results in the two exposure systems are presented in Table 1.

Table 1 (from Munoz L. M. H. *et al.* 2015, Acta Biol. Colomb., Vol. 20, No. 2, pp. 153-161). Mortality Percentage, LC_{50} and confidence intervals (CI) at 95% of the species exposed to Roundup® Activo under laboratory and microcosm conditions.

LABORATORY						
Species	**Concentration* (µg a.e. of glyphosate/L]**					**LC_{50} (CI) (µg a.e. of glyphosate/L]**
	325	**750**	**1500**	**3000**	**6000**	
R. marina	0	25	40	90	100	1423 (1129-1794)
R. humboldti	5	10	0	75	100	2437 (2095-2834)
H. crepitans	20	10	55	100	100	1414 (1157-1728)
E. pustulosus	0	5	10	45	100	2789 (2295-3389)
MICROCOSMS						
Species	**Concentration [kg a.e. of glyphosate/ha]**					**LC_{50} (CI) [kg a.e. of glyphosate/ha]**
	1.845	**3.69**	**7.38**	**14.76**	**29.52**	
R. marina	2	6	8	20	100	16.9 (15.2-18.9)
R. humboldti	24	4	92	100	100	5.1 (4.9-5.4)
H. crepitans	30	48	92	96	100	4.0 (3.4-4.7)
E. pustulosus	12	0	22	98	100	9.4 (8.7-10.2)

*the units were corrected by the RMS, according to the original study.

The LC_{50} endpoints achieved for each species are presented in Table 2.

Table 2 (from Munoz L. M. H. *et al.* 2015, Acta Biol. Colomb., Vol. 20, No. 2, pp. 153-161). Mortality Percentage, LC_{50} and confidence intervals (CI) at 95% of the species exposed to Cosmo-Flux®411F under laboratory and microcosm conditions.

LABORATORY						
Species	**Concentrations in mg/L**					**LC_{50} (CI)**
	106.25	**212.5**	**425**	**850**	**1700**	**mg/L**
R. marina	0	0	15	10	85	1216 (1096-1350)
R. humboldti	5	50	65	70	100	319 (239-425)
H. crepitans	0	0	0	15	60	1457 (1114-1905)
E. pustulosus	5	5	0	25	80	1160 (853-1578)
MICROCOSMS						
Species	**Concentration**					**LC_{50} (CI)**
	205.5	**410.9**	**821.9**	**1643.9**	**3287.9**	**L/ha**
	53.1	**106.2**	**212.4**	**424.8**	**849.6**	**mg/L**
R. marina	0	4	6	2	60	2905.2 (2522-3346.7) 750.7 (651.7-864.7)
R. humboldti	14	26	78	70	100	632.3 (516.2-774.8) 163.4 (133.4-200.2)
H. crepitans	8	10	10	18	32	*
E. pustulosus	30	28	20	66	74	1304.9 (1035.2-1644.3) 337.2 (267.5-424.9)

*Values that were not calculated by the program.

The morphometric results are presented in Figure 1 and 2.

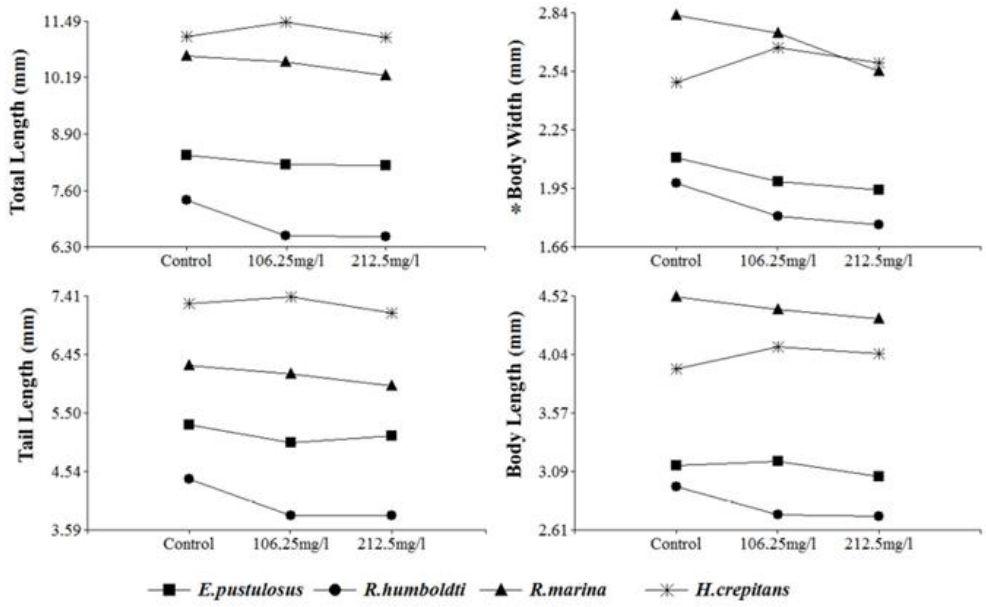


Figure 1 (from Munoz L. M. H. *et al.* 2015, Acta Biol. Colomb., Vol. 20, No. 2, pp. 153-161). Morphometric Measurement Average for the four assessed species exposed to Cosmo-Flux®411F in laboratory conditions. *There were significant differences in body width between the control and the two sub-lethal concentrations.

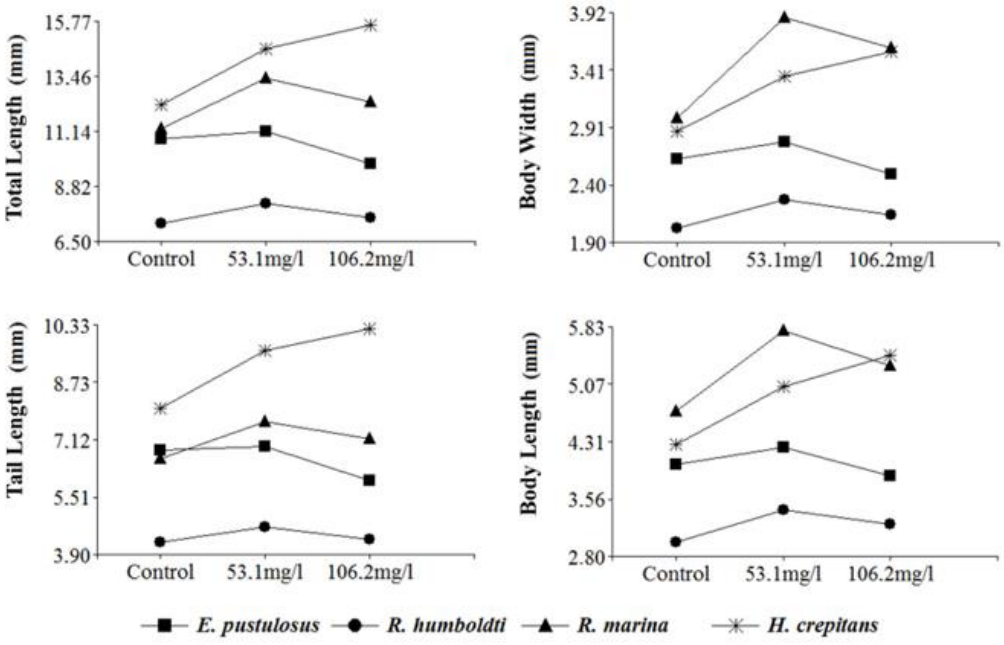


Figure 2 (from Munoz L. M. H. *et al.* 2015, Acta Biol. Colomb., Vol. 20, No. 2, pp. 153-161). Morphometric Measurement Average for the assessed species exposed to Cosmo-Flux®411F under microcosm conditions.

Conclusion

A comparison between the suggested application concentrations for the two agrochemicals with the LC50 values obtained under microcosm conditions, which better reflect actual field conditions, Roundup® Activo can cause a mortality rate of up to 50% in two of the four assessed species, showing that it is of moderate risk, while Cosmo-Flux®411F is not lethal. Laboratory and microcosm data also confirms the difference in the toxic effects of these two agrochemicals, where Roundup® Activo was approximately 700 times more toxic than Cosmo-Flux®411F.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested (Roundup Activo) is based on the potassium salt of glyphosate and contains a different surfactant system compared with the representative formulation for the glyphosate EU renewal. The article is therefore not relevant to the ecotoxicology risk assessment for the renewal as effects cannot be related to the representative formulation. In addition, the findings in this study do need to be treated with a degree of caution, as some of the presented data differs to the values discussed in the results.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

The following laboratory-generated endpoints on anuran tadpoles were obtained in the study: Roundup® Active 96 h LC_{50} ranging 1.414-2.789 mg a.e./L.

The RMS considers this study to be reliable with restrictions, since no analytical confirmation of test concentrations was performed.

Data point	CA 9
Report author	Triana Velasquez T. M. *et al.*
Report year	2013
Report title	Lethal and Sublethal Effects of Glyphosate (Roundup® Active) to Embryos of Colombian Anurans Original Title: Efectos letales y subletales del glifosato (Roundup® Activo) en embriones de anuros colombianos
Document source	Acta Biologica Colombiana (2013), Vol. 18, No. 2, pp. 271-278
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The objective of this work was to determine the lethal (median lethal concentration - LC_{50}) and sublethal effects (changes in body size and development) of glyphosate (Roundup® Active) on embryos of four

species of anurans exposed during 96 hours in laboratory and microcosm tests. In the laboratory, the more tolerant species was *Engystomops pustulosus* (LC_{50} = 3033.18 µg a.e./L) and the most sensitive was *Rhinella marina* (LC_{50} = 1421.46 µg a.e./L), which showed a significant reduction in body size and delays in the development of subjects. Other species had an intermediary LC_{50} (*Rhinella humboldti* = 2899.36 µg a.e./L; *Hypsiboas crepitans* = 2151.88 µg a.e./L). In all cases, the LC_{50} was lower than the concentration used in the field (5392.92 µg a.e./ L) indicating a high toxic effect. In the microcosm test, the embryos of E. pustulosus were the most tolerant (LC_{50} = 19.41 kg.e./ha), whereas those of *R. humb*oldti were more sensitive (LC_{50} = 10.61 kg.e./ha). However, all species had a LC_{50} higher than the concentration used in the field (3.69 kg a.e./ ha), showing a slight toxic effect and there were not any differences in the size of the body or in the development of subjects.

Materials and methods

This work was carried out with embryos of four species of anurans: *Rhinella humboldti, Rhinella marina, Hypsiboas crepitans and Engystomops pustulosus*. These species were selected because they can be found in areas that are cultivated with glyphosate, because they are not threatened and because a high number of eggs of them are laid. The embryos were obtained from different clutches of eggs around the state of Tolima, Colombia.
The glyphosate used in this work was the commercial product Roundup® Active, which has a concentration of 363 g/L of glyphosate acid of formulation at 20 °C, equivalent to 446 g/L of potassium salt of glyphosate. In addition, the commercial presentation mentions that it has additive ingredients, c.s.p. 1 L, but it does not describe them. The experimental solutions of the herbicide were prepared with water previously dechlorinated by aeration and applied in laboratory and microcosm experiments.

Twenty-five embryos in stage ten of each species, plus their counterpart from a different clutch of eggs, were exposed to each one of five experimental concentrations of glyphosate (325; 750; 1500; 3000 and 6000 µg a.e./L) and one negative control (dechlorinated water) in 2 L fish tanks with 1 L of the solution.

Fish tanks were placed in an area of the laboratory under controlled conditions of temperature (24 ± 2°C) and a light-dark cycle of 12:12 h, maintained with lamps of white light and a digital timer. The experiment consisted of a semi-static system, where solutions were renewed daily in order to maintain the concentrations of glyphosate, which are reduced after 24 h. During the replacement, pH, dissolved oxygen, conductivity and temperature were measured before and after the renewal.

Fifty embryos in stage ten for each species, plus their counterpart from several different clutches of eggs (total of embryos = 100) were exposed to each one of five experimental concentrations of glyphosate (1.845 kg a.e./ha; 3.69 kg a.e./ha; 7.38 kg a.e./ha; 14.76 kg.e./ha and 29.52 kg.e./ha), plus one negative control (chlorinated water) for 96 h. The concentrations are expressed in units of acid kilogram equivalent per hectare for comparison with the units of field application.

The observations of mortality were made at 24, 48, 72 and 96 h after the beginning of the test. The cumulative mortality up to 96 h was used to estimate the values of LC_{50}. This estimate, with its confidence intervals at 95%, was made by means of the program Trimmed Spearman-Karber (TSK) version 1.5.

To assess the sublethal effects, at the end of the experiment (96 h), photos were taken of 20 surviving subjects (already as tadpoles) for each concentration and for the control, and with the help of program ImageJ1.42, the total length, body length, tail length and width of the head were all measured. These data were analyzed by means of a multivariate analysis of variance (Manova) and subsequent analysis of variance (Anovas) so as to establish the morphometric differences of subjects between treatments.

Results

The results of the LC_{50} for the four study species (Fig. 1A, Table 1) show that R. marina was the most sensitive species, while *E. pustulosus* was the most resistant one and *H. crepitans* presented an intermediate fatality rate. When comparing confidence intervals (Fig. 1A), only *E. pustulosus* and *R.*

humboldti did not differ statistically. Mortality under field conditions as simulated in the mesocosm study are presented in Fig. 1B and Table 1.

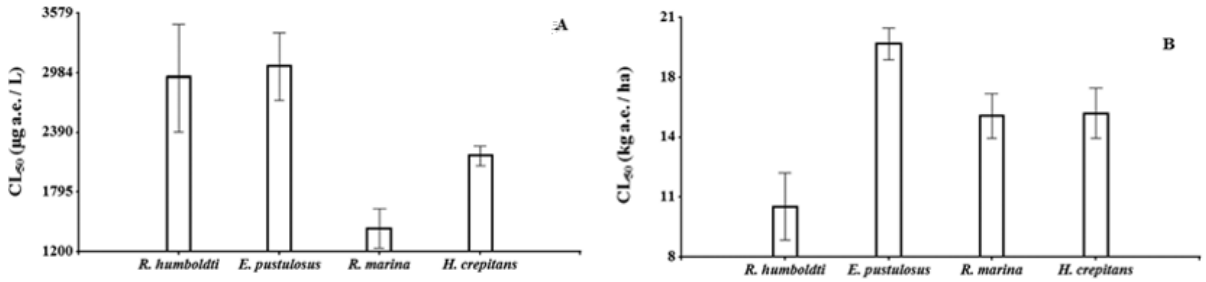


Figure 1 (from Triana Velasquez T. M. *et al.* 2013, Acta Biol. Colomb., Vol. 18, No. 2, pp. 271-278). Comparison of toxicity values (LC_{50}) in the embryos exposed to glyphosate (Roundup® Active) under laboratory (A) and microcosm (B) conditions. The bars indicate the confidence intervals at 95 %.

Table 1 (from Triana Velasquez T. M. *et al.* 2013, Acta Biol. Colomb., Vol. 18, No. 2, pp. 271-278). Mortality percentage of embryos exposed for 96 h to the individual application of glyphosate (Roundup® Active) under laboratory and microcosm conditions.

Concentrations in laboratory (µg a.e. / L)						
Species	**Control**	**[325]**	**[750]**	**[1500]**	**[3000]**	**[6000]**
E. pustulosus	0	0	8	0	40	100
R. humboldti	0	24	24	22	46	100
R. marina	0	2	26	30	100	100
H. crepitans	0	6	2	2	94	100
Concentrations in microcosm (kg a.e. / ha)						
Species	**Control**	**[1.845]**	**[3.69]**	**[7.38]**	**[14.76]**	**[29.52]**
E. pustulosus	0	5	0	0	10	100
R. humboldti	0	17	18	39	53	100
R. marina	4	4	14	2	37	100
H. crepitans	0	10	10	9	39	100

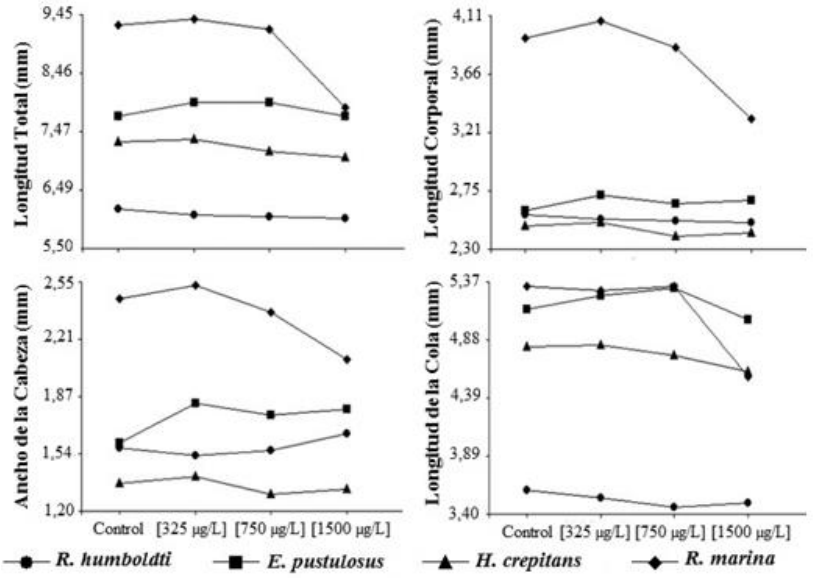


Figure 2 (from Triana Velasquez T. M. *et al.* 2013, Acta Biol. Colomb., Vol. 18, No. 2, pp. 271-278). Means of the morphometric measurements of tadpoles of the four species of surviving anurans to the concentrations of glyphosate (Roundup® Active) under laboratory conditions.

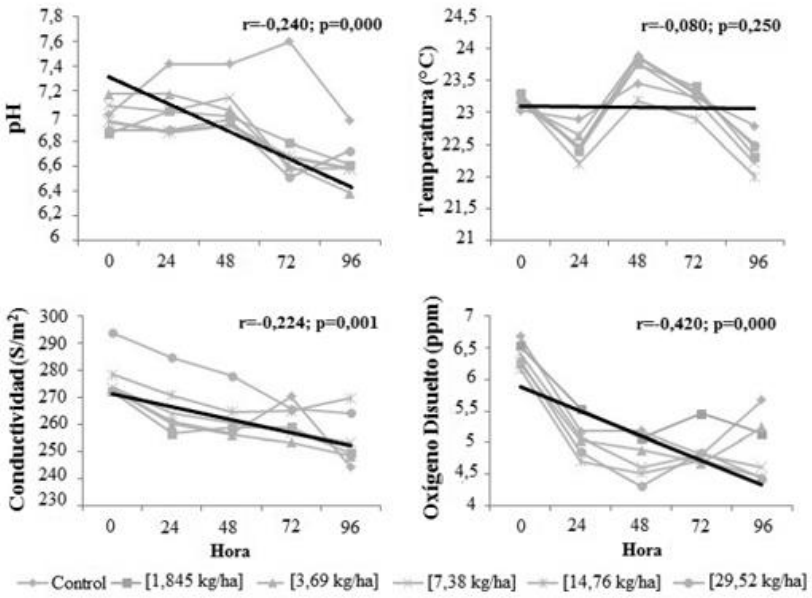


Figure 3 (from Triana Velasquez T. M. *et al.* 2013, Acta Biol. Colomb., Vol. 18, No. 2, pp. 271-278). Trend of the physico-chemical parameters on the trials of exposure to glyphosate (Roundup® Active) under microcosm conditions in the four study species. The black line represents the general trend for each parameter during the 96 h of the trial.

The laboratory trials suggest a high toxicity for glyphosate (Roundup® Active) in the embryos of anurans, but the microcosm trials indicate that it's at the suggested field concentrations, would have cause a fatality rate of around 20% without causing effects on body size nor in the development of surviving subjects.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested in this paper (Roundup® Active, 363 g/L of glyphosate acid, equivalent to 446 g/L of potassium salt of glyphosate) is not the representative formulation for the glyphosate EU renewal. The local species tested in Colombia are of questionable relevance to an EU evaluation since the data can be difficult to extrapolate to EU (e.g. local native species, geo-climatic properties, land-uses and agricultural practices differing from EU).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning regarding the non-relevance of this study. There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach.

Although there is no specific information on the prior exposure history of the sampled organisms, the authors state that the test species are generally tolerant to glyphosate. Moreover, any potential pre-exposure would also be accounted for by the control organisms, which were collected from the same

locations as the treated ones. The RMS notes some differences in experimental design (number of test embryos, temperature and renewal of test media) between the laboratory and microcosm experiments.

The laboratory experiment resulted in 96 h LC_{50} values ranging 1421-3033 µg a.e./L.

For the microcosm experiment, the LC_{50} values ranged 10.61 - 19.41 kg.e./ha. Based on the surface area treated (0.38 m2), and the total water volume in the microcosms (10 L), these application rates correspond to nominal concentrations ranging 40.8-74.7 mg a.e./L.

The RMS considers this study to be reliable with restrictions since no analytical confirmation of test concentrations was performed.

Data point	CA 9
Report author	Brodeur J. C. *et al.*
Report year	2014
Report title	Synergy between glyphosate and cypermethrin based pesticides during acute exposures in tadpoles of the common South American toad *Rhinella arenarum*
Document source	Chemosphere, (2014) Vol. 112, pp. 70 6
Short description of literature article	The current study evaluated the toxicity of equitoxic and nonequitoxic binary mixtures of glyphosate and cypermethrin based pesticides to tadpoles of the common South American toad, *Rhinella arenarum*. Two different combinations of commercial products were tested: glyphosate Glifosato Atanor + cypermethrin Xiper_and glyphosate Glifoglex + cypermethrin Glextrin When tested individually, the formulations presented the following 96 h LC_{50}s: Glifosato Atanor 19.4 mg a.e./L and Glifoglex 72.8 mg a.e. /L, Xiper 6.8 mg/L and Glextrin 30.2 mg/L. Equitoxic and non equitoxic mixtures were significantly synergic in both combinations of commercial products tested. The magnitude of the synergy (factor by which toxicity differed from concentration addition) was constant at around twofold for all tested proportions of the glyphosate Glifoglex + cypermethrin Glextrin mixture; whereas the magnitude of the synergy varied between 4 and 9 times in the glyphosate Glifosato Atanor + cypermethrin Xiper mixture.
Short description of findings	The current study demonstrates that glyphosate and cypermethrin based pesticides exhibit synergic interactions during acute exposures in tadpoles of the common South American toad, *R. arena*rum. Given the widespread use glyphosate and cypermethrin in soy and corn cultures, it is likely that these two pesticides will occasionally co occur in ephemeral ponds or aquatic systems supporting amphibian wildlife. In view of the current amphibian declines and their close association to agriculture, the findings obtained in the current study urge for more research to be promptly undertaken in order to understand the mechanisms behind the synergy observed and to identify and quantify the extent of its environmental impacts.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Observations is caused by mixture of compounds/ potentially causal factors and thus not attributable to a

	substance of concern. The glyphosate-based formulation is not the representative formulation for the glyphosate EU renewal and thus the article is not relevant. **Further points of clarification:** The representative formulation is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	The RMS does not agree with the applicant's justification. There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach. Exposure of *Rhinella arenarum* to glyphosate-based formulations resulted in 96 h LC_{50} values of 19.4 mg a.e./L for Glifosato Atanor and 72.8 mg a.e./L for Glifoglex. The RMS considers this study to be reliable.

Data point	CA 9
Report author	Navarro Martín L *et al.*
Report year	2014
Report title	Effects of glyphosate based herbicides on survival, development, growth and sex ratios of wood frogs (*Lithobates sylvaticus*) tadpoles. I:chronic laboratory exposures to VisionMax®
Document source	Aquatic Toxicology, (2014) Vol. 154, pp. 278 90
Short description of literature article	The purpose of this study was to determine if chronic exposure to the glyphosate based herbicide VisionMax affects the survival, development, growth, sex ratios and expression of specific genes involved in metamorphosis of wood frog tadpoles (*Lithobates sylvaticus*). Tadpoles were chronically exposed in the laboratory from Gosner developmental stage 25 to 42 to four different concentrations of VisionMax (0.021, 0.55, 1.1 and 2.9 mg acid equivalents/L).
Short description of findings	Chronic exposures to VisionMax had direct effects on the metamorphosis of *L. sylvaticus* tadpoles by decreasing development rates, however, there was a decrease in survival only in the group exposed to the highest dose of VisionMax (2.9 mg a.e./L; from approximately 96% in the control group to 77% in the treatment group). There was a decrease in the number of tadpoles reaching metamorphic climax, from 78% in the control group to 42% in the VisionMax (2.9 mg a.e./L) group, and a 7 day delay to reach metamorphic climax in the same treatment group. No effects of exposure on sex ratios or gonadal morphology were detected in tadpoles exposed to any of the concentrations of VisionMax tested. Gene expression analyses in brain and tail tissues demonstrated that exposure to VisionMax alters the expression of key genes involved in development. Results showed significant interaction (two way ANOVA, $P < 0.05$) between developmental Gosner stage and treatment in brain corticotropin releasing factor, deiodinase type II (dio2) and glucocorticotiroid receptor (grII) and tail dio2 and grII.

	This demonstrates that mRNA levels may be differently affected by treatment depending on the developmental stage at which they are assessed. At the same time there was a clear dose response effect for VisionMax to increase thyroid hormone receptor β in tadpole brain ($F(2,69) = 3.475$, $P = 0.037$) and tail ($F_{(2,69)} = 27.569$, $P < 0.001$), regardless of developmental stage. Delays in development (or survival) were only observed in the group exposed to 2.9 mg a.e./L of VisionMax®, suggesting that tadpoles need to be exposed to a "threshold" concentration of glyphosate based herbicide to exhibit phenotypic observable effects.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: The formulation tested is not the representative formulation for the glyphosate EU renewal, therefore, article is not relevant. **Further points of clarification:** The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach. VisionMax® at a concentration of 2.9 mg a.e./L decreased survival, development rates and metamorphic success of *L. sylvaticus* tadpoles. The RMS proposes that the NOEC for these effects can be set to 1.1 mg a.e./L. In addition, the observed effects on development rate and metamorphosis may be relevant for the ED assessment. The RMS considers this study to be reliable.

Data point	CA 9
Report author	Poletta G. L. *et al.*
Report year	2011
Report title	Genetic, enzymatic and developmental alterations observed in *Caiman latirostris* exposed *in ovo* to pesticide formulations and mixtures in an experiment simulating environmental exposure
Document source	Ecotoxicology and Environmental Safety, (2011) Vol. 74, No. 4, pp. 852 9
Short description of literature article	The aim of this study was to determine the effects of pesticides formulations and mixtures on a South American caiman, *Caiman latirostris*, after *in ovo* exposure. A field like experiment was conducted which simulates the environmental exposure that a caiman nest can receive in neighbouring croplands habitats. Experimental groups were Control group, Treatment 1: sprayed with a glyphosate herbicide formulation, and Treatment 2: sprayed with a pesticide mixture of glyphosate, endosulfan and cypermethrin formulations.

	Glyphosate based herbicide Roundups Full II (66.2% glyphosate; containing potassium salt) was tested.
Short description of findings	Field like exposure to the single glyphosate formulation Roundups Full II and to the mixture of glyphosate, endosulfan and cypermethrin formulations in conditions commonly applied in agricultural practices, induced genotoxic alterations, growth delay as well as enzymatic and metabolic disorders in caimans exposed in ovo. All parameters analysed indicated a higher toxicity for the mixture of pesticides than for Roundups Full II formulation alone. Genotoxic, enzymatic and metabolic biomarkers as well as growth parameters demonstrated to be good early indicators of contamination with pesticide single compounds and mixtures in *C. latirostris*.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). No data presented relevant for the EU ecotoxicological regulatory risk assessment. Observations are caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity). In addition, the tested formulation is not the representative formulation for the glyphosate EU renewal. **Further points of clarification:** The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach. Spraying caiman eggs with a 17.25 g/L glyphosate solution caused a significant reduction in total length and SVL at birth and at 3 months, as well as an increase in alanine aminotransferase, micronucleus frequency and damage index. Only one treatment level was tested, hence no NOEC could be determined from this study. The RMS considers this study to be reliable.

Data point	CA 9
Report author	Relyea R. A.
Report year	2018
Report title	The interactive effects of predator stress, predation, and the herbicide Roundup
Document source	Ecosphere, (2018) Vol. 9, pp. e02476
Short description of literature article	As the number of studies examining the effects of contaminants grows, ecologists are becoming increasingly aware that contaminants can interact with natural stressors (e.g., competition and predator

	cues) in their effects on non-target animals. In amphibians, predator cues can make contaminants more lethal under laboratory conditions, but the opposite outcome has been observed under more natural conditions with stratified water columns; stratification causes more pesticide to be present near the surface while predator cues scare spring breeding amphibians down to the benthos. The aim of the study was to examine whether this phenomenon also occurs in three species of summer breeding amphibians (*Hyla versicolor, Rana clamitans, and Rana catesbeiana*) that were raised in outdoor mesocosms. Specifically, it was observed how amphibian survival was affected by multiple concentrations of a common herbicide Roundup Original Max (EPA Registration No. 524–539; 540 g a.e./L), the herbicide combined with chemical cues from predators (caged larval dragonflies; *Anax junius*), and the herbicide combined with lethal predators.
Short description of findings	Environmentally relevant concentrations of the herbicide caused high rates of tadpole mortality, but this outcome was substantially reversed by the addition of predator cues. With lethal predators, the tadpoles experienced such high mortality that the herbicide caused no additional effect. Roundup Original Max also induced morphological changes in *Hyla versicolor*, and the induced traits were different from those induced by predators. Collectively, these results suggest that while predator cues can make pesticides less lethal when thermal stratification occurs, highly lethal predators can overwhelm these effects. Thus, the impacts of such contaminants can be dramatically different in environments that do or do not contain high risk predators.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: The tested formulation is not the representative formulation for the glyphosate EU renewal; therefore, the paper is not relevant to the EU renewal. **Further points of clarification:** The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	The LC_{50} values are 2.3 mg a.e./L for *Hyla versicolor* and 3 mg a.e./L for *Rana catesbeiana*, while mortality of *Rana clamitans* did not reach 50%. NOEC for survival of tadpoles from all 3 species is 2 mg a.e./L. NOEC for body mass of *Hyla versicolor* tadpoles is also 2 mg a.e./L. These values represent exposure of the tadpoles without predators. There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation. Therefore, the study is considered less relevant but supplementary and will be used in a WoE approach. The RMS considers this study to be relevant and reliable.

Data point	CA 9
Report author	Slaby S. *et al.*
Report year	2019

Report title	Effects of glyphosate and a commercial formulation Roundup exposures on maturation of *Xenopus laevis* oocytes
Document source	Environmental Science and Pollution Research, (2019) Ahead of Print. https://doi.org/10.1007/s11356 019 04596 2
Short description of literature article	The aim of this work is to characterize the effects of glyphosate and its commercial formulation Roundup GT Max on the *Xenopus laevis* oocyte maturation, which is an essential preparation for the laying and the fertilization. Kinetics of the maturation process was assessed by determining GVBD (Germinal Vesicle Breakdown) ratios every 15 min for 13 h. Test item: Roundup GT Max contains 480 g glyphosate acid/L (588 glyphosate potassium salt/L).
Short description of findings	*GVBD time courses* Glyphosate exposure with Pg: Proportions of oocytes with a WS significantly decreased ($p < 0.05$) after 420 and 435 min of exposure to 14.8 and 148 µM of glyphosate, but these delays quickly disappeared and were not observed again until the end of the experiments. At 1480 µM of glyphosate, more important delays were recorded. GVBD ratios were significantly lower after 375 ($p < 0.01$), 390 ($p < 0.05$), 405 to 450 ($p < 0.01$), 465 to 570, and 695 to 785 ($p < 0.05$) minutes. The last ratio that measures at 800 min was not significantly different from the control condition. Roundup GT Max exposure with Pg induced approximatively the same response profiles, but no significant difference was found after statistical analyses. Without Pg, spontaneous GVBD were observed in glyphosate and Roundup GT Max conditions and especially for the concentration 1480 µM [a.e.] of Roundup GT Max where GVBD ratios were significantly higher from 570 min to the end of the exposure. *Meiosis spindle formation analyses* Control immature oocytes show at the cytological level a large germinal vesicle. After the GVBD, in a control mature oocyte, there is the formation of the meiotic spindle. This barrel shaped structure close to the cell membrane supports the condensed chromosomes lined up on the metaphase plate. Abnormal structures of the meiotic spindle formation can be observed in the control mature oocytes (30.10%). For the Roundup GT Max concentration of 1480 µM [a.e.], this percentage significantly increased to 76.19% ($p < 0.05$). A similar increase was observed for the same concentration of glyphosate (56.25%) but did not differ significantly from the control condition ($p = 0.0572$). *MAPK and MPF activity investigations* No particular abnormality in the phosphorylation states of ERK2, RSK, Cdc2, and H3 was detected on the immunoblots. Only one experiment exhibited an aberrant phosphorylation state in pooled white spotted oocytes of Cdc2 (glyphosate 1480 µM) and H3 (Roundup GT Max 1480 µM [a.e.]). This suggested that the MAPK pathway and also the activation and the activity of the MPF were not impacted by glyphosate or Roundup GT Max exposure. The results showed that exposures to both forms of glyphosate delayed the hormone dependent process and were responsible for

	spontaneous maturation. Severe and particular morphogenesis abnormalities of the meiotic spindle were also observed. The MAPK pathway and the MPF did not seem to be affected by exposures. The *Xenopus* oocyte is particularly affected by the exposures and appears as a relevant model for assessing the effects of environmental contamination.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: The formulation tested is not the representative formulation for the glyphosate EU renewal. In addition, enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological regulatory risk assessment. **Further points of clarification:** The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation. Moreover, although not relevant for the risk assessment, the effects at suborganismal level investigated in this study can provide useful information for the ED assessment. The RMS considers this study as reliable with restrictions, since no analytical confirmation of the test concentrations was performed.

The RMS proposes that the following studies are also considered as potentially relevant for the assessment of direct effects on amphibians. For detailed summaries, please refer to the Appendix of Vol. 3CP Section B.9 Published literature for the biodiversity assessment. The respective endpoints are listed in Volume 1.

Edge C, Gahl M, Pauli B, Thompson D and Houlahan J 2011 - Exposure of juvenile green frogs (*Lithobates clamitans*) in littoral enclosures to a glyphosate-based herbicide.

Edge C, Gahl M, Thompson D and and Houlahan J 2013 - Laboratory and field exposure of two species of juvenile amphibians to a glyphosate-based herbicide and *Batrachochytrium dendrobatidis*.

B.9.2. EFFECTS ON AQUATIC ORGANISMS

B.9.2.1. Acute toxicity to fish

B.9.2.1.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.2.1/021
Report author	Antunes, A. M. *et al.*
Report year	2017
Report title	Gender-specific histopathological response in guppies *Poecilia reticulata* exposed to glyphosate or its metabolite aminomethylphosphonic acid

Document No	DOI 10.1002/jat.3461
Guidelines followed in study	None
Deviations from current test guideline identified by the applicant: See RMS analysis in RMS comment box	*Not applicable*
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Yes, reliable with restriction

Summary of the study according to OECD format

Ecotoxicity of glyphosate (GLY) and its metabolite aminomethylphosphonic acid (AMPA) was investigated in guppies, *Poecilia reticulata*. The median lethal concentration after 96 hours of exposure (LC_{50}, 96 h) of both test item was determined in male and female guppies

Both genders showed similar median lethal concentration (LC_{50}) at 96 hours for glyphosate and AMPA. The acute 96 hour-LC_{50} of glyphosate obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L) and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively. The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

Materials and methods

Tested products; GLY and AMPA 96% and 99%, respectively, were purchased from Sigma-Aldrich (São Paulo, SP, Brazil). The stock solutions of GLY and AMPA were prepared in ultrapure water with a nominal concentration of 250 mg l^{-1}.

Animal collection and maintenance; *P. reticulata* used in the experiments was part of the animals group kept in the Aquatic Animal Biotery of the Cell Behavior Laboratory (Institute of Biological Sciences IV, Universidade Federal de Goiás, Goiânia, Brazil). All of them were 3-month-old F1 generation animals born in the biotery from a wild parental generation. 318 mature male and 318 mature female guppies (vitellogenic oocyte occurred) of an average weight of 252 ± 20 mg and 178.6 ± 14.4 mg, and total average length of 2.98 ± 0.3 cm and 2.47 ± 0.2 cm, respectively.

Toxicity test: LC_{50}; For each experimental condition, eight males or eight females guppies were maintained in 2 liter tanks (4 fish/L) and exposed to different nominal concentrations of GLY (50, 55, 60.5, 66.5 and 73.2 mg/L) or AMPA (86.8, 104.2, 125, 150 and 180 mg/L) during 96 h in the static test under 12: 12 h light/dark cycles. These concentrations were determined in the preliminary tests. The control group that consisted of eight fish kept in dechlorinated water. All treatments were performed in a triplicate design and the fish were not fed during the experimental period (USEPA, 1993). The mortality was reported at different exposure times (2, 4, 6, 8, 10, 12, 24, 48, 72 and 96 h). Physical and chemical parameters of water were analyzed every morning and did not show any changes over the experimental period, such as temperature 24 ± 1 °C, dissolved oxygen 8 mg/L, ammonia 0.002 mg/L, pH 7.0 ± 1, nitrite 0.025 mg/L and nitrate 0.5 mg/L. Cumulative mortality data obtained at the end of the experiments (96 h) were analyzed by the trimmed Spearman–Karber method to estimate the LC_{50} of a 96 h exposure to GLY and AMPA.

Statistical analysis; All statistical analyses were performed using the Statistica 7.0 software (Statsoft Inc., 2005, Tulsa, OK, USA). The differences between the treatments of the analyzed variables were

identified using parametric tests (two-way ANOVA, followed by the Tukey's test) and/or non-parametric tests (Kruskal–Wallis), depending on the distribution of the data and homogeneity of variances (Shapiro–Wilk and Levene's tests). Linear and non-linear regression analyses were also applied to verify the relationship between variables.

Results

Median lethal concentration (LC_{50}); No mortality was observed for both genders in the control group during the experimental period of 96 h. The LC_{50} results showed that the GLY is more toxic to the guppies than to its metabolite AMPA, whereas no significant difference was observed between the genders ($P > 0.05$). The GLY LC_{50} values obtained for male and female *P. reticulata* were, respectively, 68.78 mg/L (95% confidence interval = 64.59–73.24 mg/L) and 70.87 mg/L (95 % confidence interval = 65.91–76.26 mg/L). The AMPA LC_{50} in turn, were 180 mg/L (95 % confidence interval = 175.12–184.54 mg/L) and 164.3 mg/L (95 % confidence interval = 160.6–168.54 mg/L), respectively. The GLY and AMPA toxicity increased linearly with the increasing concentration for females (GLY: $y = 0.6281x – 30.141$, $r = 0.96$, $P < 0.05$; AMPA: $y = 0.150x – 11.193$, $r = 0.93$, $P < 0.05$) and males (GLY: $y = 0.666x – 30.653$, $r = 0.93$, $P < 0.05$; AMPA: $y = 0.168x – 11.898$, $r = 0.88$, $P < 0.05$).

Discussion

The results of the LC_{50} values of GLY (male 68.78 mg/L and female 70.87 mg/L) and AMPA (male 180 mg/L and female 164.3 mg/L) based on the mortality test indicated a low sensitivity of *P. reticulata* in comparison to the other teleost species, as reported by the USEPA. In addition, it was observed that the AMPA is less toxic to *P. reticulata* than GLY (male 2.6-fold, female 2.3-fold).

Conclusion

The present study determined the acute 96 hour-LC_{50} of glyphosate and AMPA. The glyphosate LC_{50} values obtained for male and female guppies *P. reticulata* were 68.78 mg/L (95 % C.I.: 64.59–73.24 mg/L and 70.87 mg/L (95 % C.I.: 65.91–76.26 mg/L), respectively.
The 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L (95 % C.I.: 175.12–184.54 mg/L) and 164.3 mg/L (95 % C.I.: 160.6–168.54 mg/L), respectively.

Assessment and conclusion by applicant:

The acute 96 hour-LC_{50} values for male and female guppies *P. reticulata* after exposure to glyphosate were 68.78 mg/L and 70.87 mg/L, respectively. The acute 96 hour-LC_{50} values for AMPA for male and female guppies were 180 mg/L and 164.3 mg/L, respectively.
In the material and methods part some important is missing. No information on preparation of test solution and application is given. Source and composition of media are unclear. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

Assessment and conclusion by RMS:
No information on methods of preparation of stock and test solutions was reported.

No analytical verifications of test concentrations were reported.

According to OECD 203 (2019) for the assessment of acute mortality, test fish must be juveniles (i.e. before reaching sexual maturity) whatever the species tested. This was not the case in this study (not conducted according to OECD guideline) as mature individuals were used. The relative sensitivity of individuals of this age is not known.
The sensitivity of juveniles might not be covered by this study.

Sublethal concentrations of glyphosate and metabolite AMPA induced severe damage to the liver and gills of the guppies. Morphological changes on gills seem to be defense responses in the gills (proliferation of the interlamellar epithelium, partial/total fusion of the secondary lamellae, edema). The study suggests they may affect the breathing process leading to hypoxia. Histopathological changes in gills were similar for the males and females.

The liver showed mainly regressive changes, such as steatosis, pyknotic nuclei and high distribution of collagen fibers. The liver response was different between the genders. The hepatic inflammatory changes were more common in males.

This study suggests that the toxic effect of pollutants in aquatic species must not be assessed only by mortality rates, but the reproduction and growth endpoints should also be taken into consideration, as they are harmful to the animal's health.

The study is considered as relevant and reliable with restriction.

Following endpoints are considered as supportive only:
Acute 96 hour-LC50 values for male and female guppies *P. reticulata* after exposure to glyphosate = 68.78 mg/L and 70.87 mg/L, respectively.
Acute 96 hour-LC50 values for AMPA for male and female guppies = 180 mg/L and 164.3 mg/L, respectively.

Data point:	CA 8.2.1/022 CA 8.2.1/023
Report author	Gholami, S.J. *et al*.
Report year	2013
Report title	Toxicity evaluation of Malathion, Carbaryle and Glyphosate in common carp fingerlings (*Cyprinus carpio*, Linnaeus, 1758)
Document No	ISSN: 2008-2525
Guidelines followed in study	OECD 203
Deviations from current test guideline identified by the applicant: **See RMS analysis in RMS comment box**	*None*
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability: **as provided in the AIR5 dossier (KCA 9) as proposed by applicant:** **See RMS analysis in RMS comment box**	Yes, reliable with restriction

Summary of the study according to OECD format

Fingerlings of the common carp (*Cyprinus carpio*, Linnaeus, 1758) are often exposed to a wide range of pesticides when they are released introduced into the sea at the estuaries of the rivers flowing into the Caspian Sea. The present study investigated effects of lethal concentrations (expressed as 96-hour LC_{50s})

and sublethal concentrations (determined by acetylcholinesterase assay) of glyphosate on these fingerlings.
The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.
These endpoints are considered as only supportive by RMS as some validity criteria could not be checked and no analytical verifications were reported.

Materials and methods
Chemicals: Sodium carbonate, sodium hydroxide, copper sulfate, potassium sodium tartrate, bovine serum albumin, phosphoric acid, tris and hydrochloric acid were purchased from the official representative of the German Company Merck in Iran. Glyphosate was purchased from Bazargan Kala (Iran). Absorbance was read using an ELISA Microplate Reader (ELx 808, BioTek).

Reactants: 0.1 mol phosphate buffer solution (PBS) (pH 7 with no Tritone), the Folin–Ciocalteu reagent (FCR) (diluted with an equal volume of distilled water), DTNB (dissolved in TRIS/HCl buffer) and acetylthiocholine iodide were used in the experiments.

Two thousand fingerlings with the mean weight of 2±0.4 g were obtained from the Shahid Rajaee Fish Breeding and Rearing Center, Sari, Mazandaran Province, and were transferred to the Fish Breeding and Rearing Research Center in the Department of Fisheries at the College of Agriculture & Natural Resources (UTCAN) in University of Tehran (Karaj). In order to adapt to the new environmental conditions, the fish were kept in two 1000-liter fiberglass tanks for 15-20 days. The physicochemical parameters of water were controlled as follows: pH=7, total water hardness ($CaCO_3$) =175 mg/l, dissolved oxygen=more than 7 ppm and temperature=20±2°C. The stock solution of glyphosate was prepared with the concentration of 10,000 ppm.

Lethal concentration experiments (bioassays): To perform bioassay, the range of concentrations of glyphosate and the logarithmic distances were determined in a pilot test and then the main experiment was carried out. Based on the results of this pilot test, the fingerlings were exposed to the following concentrations of glyphosate for 96 hours: 5.5, 6.5, 7.5, 8.5, and 9.5 mg/l. Effects and LC_{50s} were determined in accordance with the OECD Guidelines for the Testing of Chemicals (No. 203) in static water. Bioassay for each pesticide was performed on 150 fingerlings (a total of 450) that were randomly and equally put in fifteen 100-N fiberglass tanks (three replicates for each concentration with 10 fish in each tank). The experimental conditions were close to those during the adaptation period. The fingerlings were not fed during the experiment. All experimental groups were monitored twice a day and the behavior of the fingerlings was studied. Moreover, the number of deaths was recorded at 24, 48, 72, and 96 hours after the toxin was added.

Sublethal toxicity experiment: The fingerlings were randomly placed in nine 100-N fiberglass tanks. Each tank contained 40 fingerlings, and the experimental conditions were the same as in the previous experiments. As in the rearing and adaptation periods, the subjects were fed 2% of their body weight and the feeding was stopped 24 hours before they were killed. In the sublethal toxicity experiment, the fingerlings were exposed to three different concentrations of glyphosate, each with three replicates for 15 days. The treatments were as follows: 0 (control), 0.6, and 1.2 mg/l of glyphosate. These concentrations were determined based on the LC_{50} values. About 10% of the water in each tank was siphoned off every day in order to remove waste materials and reduce ammonia levels in the water. To maintain the stability of experimental conditions, the removed water was replaced by an equal volume of water with the initial concentrations of the pesticide.

Sampling and extract preparation (upper layer): A number of fingerlings from each treatment were sampled on the fifth, tenth, and fifteenth days after their first exposure to pesticides. Because of the very small size of the fingerlings, it was not possible to take blood or tissue samples. Therefore, they were beheaded and both parts (head and trunk) were frozen at -70°C to be later used for extract preparation. The obtained tissues were manually homogenized in 0.1mmol PBS (pH 7 and containing 1% of Tritone X-100). The samples were centrifuged and the resulting extract (upper layer) was removed to be used as the enzyme source.

Total protein assay and ACHE activity measurement: Total protein concentration in the tissues was measured by using the Lowry method at 540 nm utilizing an ELISA microplate reader. In this method, FCR was used as the color reagent. Protein concentration in tissue samples was then determined using the resulting curve and its linear equation. The specific activity of cholinesterase (in µU/min/mg protein) was measured based on Ellman's method at 420 nm using a microplate reader. To this end, a mixture of the extract (upper layer), 0.1 mol PBS, DTNB (Ellman's reagent) and acetylthiocholine iodide were added to each tube. Finally, 100 ml of the final solution was poured into each well of the microplate and absorbance per minute (O.D. /min) was read.

Calculations and statistical analysis: The data obtained from the bioassay and mortality rate of the fingerlings determined by using the probit model were analyzed. The values obtained from bioassays were then estimated using the POLO-PC 2002 software (under license of the University of Tehran). The specific activity of the enzyme (in µU/min/mg protein) was calculated as the dependent variable. The data were statistically analyzed using two-way ANOVA. The concentrations of the pesticides and the durations of exposure to them were the independent variables. The difference between means was also evaluated using Duncan's test with type-I error level of 0.05.

Results

Bioassay results: No mortality was observed during the adaptation period of the fingerlings. The results showed that their mortality rate increased with at the higher concentrations. Based on the mortality rates in the bioassays, the mean LC_{10}, LC_{50}, and LC_{90} values of glyphosate for the fingerlings at 24, 48, 72, and 96 hours were calculated (α=0.95) (see table below).

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Table B.9.2.1-1: The mean values obtained from bioassays in Caspian Sea common carp fingerlings

Chemical's name	Lethal concentration (mg/l)	24-hour	48-hour	72-hour	96-hour
Glyphosate	LC_{10}	5.995	5.976	5.865	5.548
	LC_{50}	7.202	7.172	6.985	6.753
	LC_{90}	8.651	8.606	8.319	8.168

Conclusion

The results indicated that the 96-hour LC_{50} of glyphosate for the fingerlings was 6.75 mg/L. In addition, the lowest observed effective concentrations (LOECs) (96-hour LC_{10}) was 5.548 mg/l for glyphosate.

Assessment and conclusion by applicant:

The acute 96 hours- LC_{50} for common carp fingerlings was determined to be 6.75 mg/L by static exposure to glyphosate at 5 test concentrations between 5.5 and 9.5 mg/L.

The test was conducted according to OECD 203, but validity criteria are missing. No information on the test item such as purity is given. The results for the control are not stated. Furthermore, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

Assessment and conclusion by RMS:

Despite that the study was conducted according to OECD 203, validity criteria could not be checked (control mortality not reported). No analytical verifications were reported. Thus the study cannot be considered valid.

During the experiment, cholinesterase activity was inhibited in the fingerlings treated with sublethal concentrations of glyphosate more significantly than in those of the control group ($p<0.05$).
Respiratory disorders were observed (on fingerlings exposed to glyphosate).
This study states that durations of exposure to the pesticides had greater effects on the treated fingerlings than their concentrations.

The endpoint reported below is only supportive (relevant and reliable with restrictions, no analytics):
Acute 96 hours- LC50 for common carp fingerlings = 6.75 mg glyphosate/L

B.9.2.2. Long-term and chronic toxicity to fish

B.9.2.2.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.2.2.1/005
Report author	Rodrigues, L.B. *et al.*
Report year	2019
Report title	Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms
Document No	doi.org/10.1016/j.mrgentox.2019.05.002 E-ISSN: 1873-135X
Guidelines followed in study	OECD 236
Deviations from current test guideline identified by the applicant: See RMS analysis in RMS comment box	*Not reported*
GLP/Officially recognised testing facilities	No
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Yes/Reliable with restrictions

Summary of the study according to OECD format

The present study assessed the acute toxicity of glyphosate, as well as the main metabolite aminomethylphosphonic acid (AMPA) on non-target aquatic organisms. The toxic effects of these chemicals were evaluated in a zebrafish (*Danio rerio*) embryo-larval toxicity test according to OECD Test Guideline 236 at 6 concentrations between 1.7 and 100 mg/L. Three replicates with 20 fertilized eggs per concentration were used.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L).

Materials and methods

Test chemicals; Technical-grade glyphosate (GLY; Glyphosate PESTANAL®; purity 99%, CAS No. 1071-83-6) and aminomethylphosphonic acid (AMPA, purity 99%, CAS No. 106651-9) were purchased from Sigma-Aldrich.

Zebrafish maintenance and egg production; Adult male and female zebrafish (D. rerio) were provided by the zebrafish facility (ZebTec Tecniplast) at the Institute of Biology, University of Brasília and kept in separate tanks (ethical approval UFG Nº 102/2014). Fish were maintained in a Rack Hydrus (Alesco) recirculating system using water filtered by reverse osmosis, where water passes through several levels of filtration (activated carbon filters and biological filters), is then disinfected by ultraviolet (UV) light and automatically adjusted for pH and conductivity. The temperature was maintained at 26 ± 1 °C, conductivity at 750 ± 50 µS, pH at 7.5 ± 0.5 and dissolved oxygen of 8 ppm. Nitrate, nitrite and ammonia were regularly monitored. This water was used in preparing the test solutions of all assays performed. Adult organisms were fed with commercial dry flake food (TetraColor Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected about 30 min after natural mating, rinsed in water and examined under a stereomicroscope (Bel Photonics STM PRO). Unfertilized or damaged eggs were discarded. The fertilization success was checked, and only batches of eggs with a minimum fertilization rate of 90% were used.

Fish embryo acute toxicity (FET) test; The zebrafish embryo-larval toxicity test was carried out according to OECD Test Guideline 236. Twenty fertilized eggs per concentration were randomly selected and carefully distributed in a 24-well plate, filled with 2 mL of GLY, AMPA at 1.7, 5, 10, 23, 50 and 100 mg/L and controls (negative control – NC: maintenance water and positive control – PC: 3,4-dichloroaniline at 4.5 mg/L). Tests were performed in triplicates (three independent experiments) in a climate chamber at 26 ± 1 °C and 12 h light under static conditions. Neither food nor aeration was provided during the bioassays. Embryo development was assessed at 24, 48, 72 and 96 h post-fertilization (hpf), using a stereomicroscope (Bel Photonics STM PRO) with 3x magnification. The distinction between the normal and abnormal development of embryos was established according to the zebrafish development descriptions reported previously. Lethal (egg coagulation, no somite formation, nondetachment of the tail from yolk sac and no heart beating) and sublethal (effects on the eye and body pigmentation, absorption of the yolk sac, hatching rate, swimming bladder inflation, otolith, presence of edemas and blood accumulation, tail deformities) parameters were observed and reported.

Statistical analysis; The FET and Comet data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Each experimental value was compared to its corresponding negative control and the statistical difference was considered significant when $p < 0.05$. With respect to the FET, the toxicity was expressed as the lethal concentration (LC50), which was calculated using GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA, USA) with 95% confidence interval.

Results

Acute effects for zebrafish early-life stages; The present study investigated the effects of active ingredient GLY and its metabolite AMPA on the zebrafish embryonic development (survival and malformations) at 24, 48, 72 and 96 h of exposure. According to Fig. 1, no significant mortality was observed in zebrafish early-life stage after exposure to different concentrations (1.7–100 mg/L) of GLY and AMPA (Fig. 1), which presented survival rate ≥90% in all exposure periods.

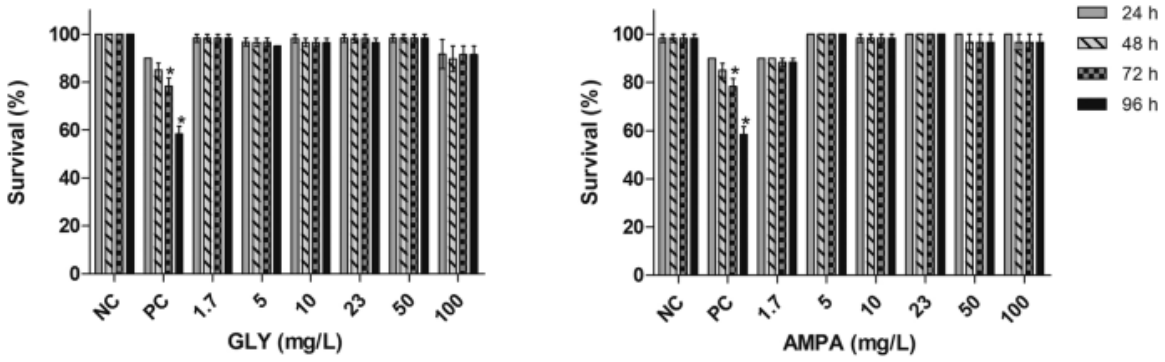


Fig. 1. Survival rate of zebrafish at different developmental stages exposed to GLY and AMPA for 24, 48, 72 and 96 h. Twenty fertilized eggs per experimental group were evaluated. Bars represent the mean ± standard error of the mean of three independent experiments. *p < 0.05 statistically different from the respective negative control (NC) based on one-way ANOVA and Dunnett's post hoc test. PC = positive control (3,4-dichloroaniline at 4.5 mg/L after 24, 48, 72 and 96 h of exposure).

In relation to sublethal effects, Fig. 2 shows that GLY induced some morphological abnormalities, however, these malformations were not statistically significant when compared to their respective negative control.

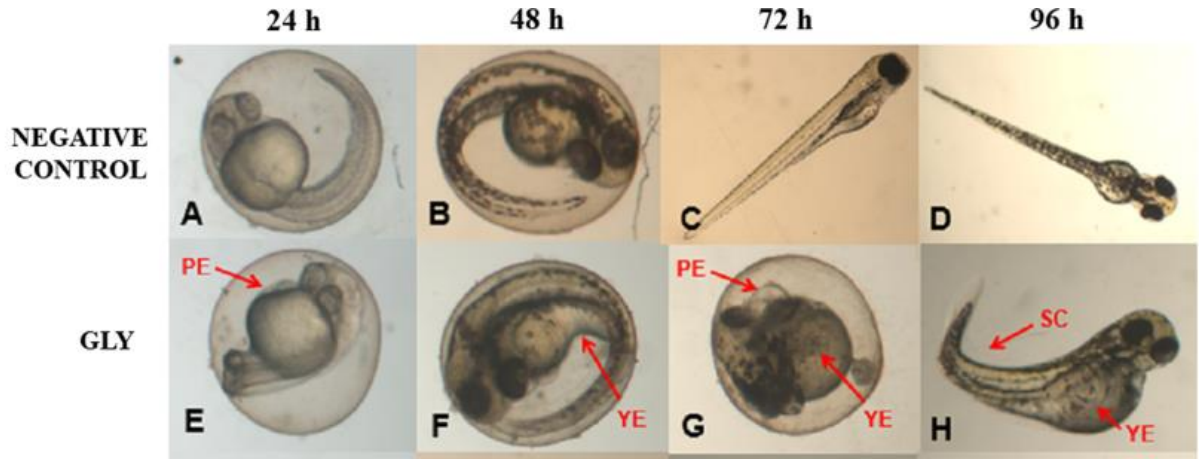


Fig. 2. Zebrafish embryos and larvae abnormalities after GLY exposure: pericardial edema (PE), yolk sac edema (YE), spinal curvature (SC). Embryos control after 24 h and 48 h of exposure, respectively (A–B); larvae control after 72 h and 96 h of exposure, respectively (C–D), embryos exposed to GLY at 23 mg/L and 100 mg/L for 24 h and 48 h, respectively (E–F); non-hatching embryo exposed to GLY at 10 mg/L for 72 h (G); larvae exposed to GLY at 100 mg/L for 96 h (H).

Discussion

The current results showed that glyphosate and AMPA did not induce acute toxicity in zebrafish early-life stage with LC_{50}-96 h > 100 mg/L.

Similar effect was observed by researchers (Roy N.M. et al, 2016) in assessing the acute effects of glyphosate (0.005; 0.05; 5; 10 and 50 mg/L) on early-life stages of zebrafish and common carp (Cyprinus carpio) for 120 h. The authors demonstrated that all tested concentrations, except the highest concentration (50 mg/L), induced cumulative mortality ≤ 10% after 96 h of exposure. Glyphosate at 50 mg/L caused the highest cumulative mortality, reaching 17.5% after 120 h of exposure while in this study, there were no significant differences between this group (glyphosate at 50 mg/L) and control with 1.7% of larvae mortality after 96 of exposure. It is worth noting that according to OECD 236, the survival of embryos in the NC must be ≥ 90% (validation criterion of the test), and therefore mortality ≤ 10% in the experimental groups is acceptable.

Conclusion

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

Assessment and conclusion

Assessment and conclusion by applicant:

The acute toxicity of technical glyphosate and its metabolite aminomethylphosphonic acid (AMPA) to zebrafish embryos was investigated.

Glyphosate and AMPA caused no acute toxic effect (LC_{50}-96 h > 100 mg/L) in zebrafish.

The study was stated to have been conducted according to OECD guideline 236, but there is no information on hatching rates in the treatment and control groups, so exposure of the embryo without a potential barrier function of the chorion cannot be confirmed.

Concerning the validity of the study, four of the six validity criteria from the test guideline are mentioned in the paper (fertilization rate of embryo batches used was >90%, survival in the negative control group was > 90%, temperature was maintained at 26 ±1ºC and dissolved oxygen was at an acceptable level 8ppm). There is no information presented on the performance of the positive control group (3, 4-dichloroaniline) and no information provided on the hatching rates in the negative control group at 96 hours, which for the control group should exceed 80%. As these information are not presented and the fact that there was no analytical verification of test concentrations reported, this study considered as reliable with restrictions.

Assessment and conclusion by RMS:

The study summary presented is only a partial summary. Indeed, only lethal effect are presented in the summary above while the main focus of this study is genotoxicity.

The relevance of the genotoxicity results for the risk assessment cannot be established by RMS as no quantitative link can be made between observations on DNA damages and the potential adverse at population level (this latter being the specific protection goal). So only results on mortality and other sublethal effects were considered in deep by RMS. Nevertheless, a link between DNA damages may exist and then may be indicative of mortality and/or potential adverse effect at population level. So, the overall conclusion of the study were also reported below (for genotoxicity part) and may be considered in future, together with other data available for the active substance.

The present study assessed the acute toxicity and genotoxicity of the glyphosate based formulation Atanor 48 (ATN) and its major constituent glyphosate, surfactant polyethoxylated tallow amine (POEA), as well as the metabolite of glyphosate AMPA, on fish.

The toxic effects of these chemicals were evaluated in the fish embryo acute toxicity test with zebrafish (*Danio rerio*), while genotoxic effects were investigated in the comet assays with cells from zebrafish larvae and rainbow trout gonad-2 (RTG-2).

Glyphosate and AMPA caused no acute toxic effect (LC50-96 h > 100 mg/L) in zebrafish.
Glyphosate induced some morphological abnormalities (from 10 mg/L to 100 mg/L), including pericardial and yolk sac edemas, spinal curvature, head and tail deformities in different exposure times; however, these malformations were not statistically significant when compared to their respective negative control.

Potential effects on hatching were not investigated. The sensitivity of the fish strain cannot be verified as no data with reference toxic 3,4-dichloroaniline was reported.

No analytical verification of test concentrations were reported, RMS considers this study as reliable with restrictions.

Additionnal information on genotoxicity (not assessed in deep by RMS):

All compounds were genotoxic in comet experiments with zebrafish larvae (LOEC 1.7 mg/L for glyphosate, ATN, AMPA and 0.4 mg/L for POEA). Unlike in vivo, only POEA induced DNA damage in RTG-2 cells (LOEC 1.6 mg/L), suggesting that it is a direct acting genotoxic agent. Genotoxic effects were observed in both RTG-2 cells (only POEA) and zebrafish (all compounds) with the lowest tested concentrations.

Data point:	CA 8.2.2.1/006
Report author	Schweizer, M. *et al.*
Report year	2019
Report title	How glyphosate and its associated acidity affect early development in zebrafish (*Danio rerio)*
Document No	DOI 10.7717/peerj.7094 ISSN: 2167-8359
Guidelines followed in study	OECD Guideline 236
Deviations from current test guideline identified by the applicant: See RMS analysis in RMS comment box	*None*
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Yes/Reliable with restrictions

Summary of the study according to OECD format

Zebrafish (*Danio rerio*) embryos exposed to concentrations between 10 µM and 10 mM glyphosate (corresponding concentrations between 1.69 and 1690.7 mg glyphosate/L) in an unbuffered aqueous medium, as well as at pH 7, for 96 hours post fertilization (hpf). Furthermore, for investigations of the influence of pH, the test concentration 1 mM glyphosate (169.07 mg glyphosate/L) was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. A total of 32 embryos were used per treatment with 8 replicates of 4 embryos each. The observed endpoints included mortality, the hatching rate, developmental delays at 24 hpf, the heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. $LC_{10/50}$, EC_{10} and, if reasonable, EC_{50} values were determined for unbuffered glyphosate.

In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 µM (LC_{10}) and 582 µM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 µM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 µM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 µM.

Materials and methods

Glyphosate; Glyphosate (N-(phosphonomethyl)glycine, 96% pure substance, molecular weight: 169.07 g/mol, CAS: 1071-83-6; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was used to prepare the test solutions. A stock solution with a concentration of 25 mM was prepared as follows: glyphosate was diluted in reconstituted water (0.23 g KCl, 2.59 g $NaHCO_3$, 4.93 g MgS_4O x 7 H_2O and 11.76 g $CaCl_2$ x 2 H_2O were dissolved separately in one L double-distilled water, then 25 mL of each stock solution was added to 900 mL double-distilled water). The stock solution was then diluted to the following test concentrations: 10, 50, 100, 250, 500, 750 µM, one and 10 mM glyphosate. All those concentrations were tested unbuffered and at pH 7. For pH adjustments, 1M HCl and NaOH solutions were used as

recommended in the Organisation for Economic Co-operation and Development (OECD) 236 (2013) guideline. For investigations of the influence of pH, 1 mM glyphosate was tested at different pH values ranging between pH 3 and 8 vs. the respective pH controls. Due to preliminary results from the broad-scale pH testing, particular attention was paid to the range between pH 3 and 4. Measurements of pH were conducted with a pH meter (SevenCompactDuo; Mettler Toledo, Gießen, Germany) directly prior to the exposure.

Maintenance of zebrafish and test procedure; The embryos used in this study stem from our own breeding stock of the D. rerio West aquarium strain established in the Animal Physiological Ecology group, Tübingen University. Adult zebrafish were kept in 90 L aquaria filled with a 1:1 mixture of purified water and filtered tap water (AE-2L water filter with an ABL-0240-29 activated carbon filter, 0.3 mm; Reiser, Seligenstadt, Germany) at 26 ± 1 °C and an oxygen saturation of 100% ± 5%. Conductivity ranged from 260 to 350 mS/cm, nitrite and nitrate concentrations from 0.025 to 0.1 mg/L, one and five mg/L, respectively, and total water hardness from eight to 12 dH. Fish were subjected to an artificial 12:12 h day/night cycle and fed three times daily with flake food (TetraMin; Tetra GmbH, Melle, Germany) supplemented with frozen black mosquito larvae and glass worms (Poseidon Aquakultur Freeze, Ruppichteroth, Germany) prior to spawning to ensure sufficient dietary protein. The day before the test, pre-exposure and test Petri dishes (90 and 30 mm in diameter) were filled with the respective solutions and stored at 26 ± 1 °C overnight to saturate the glass (the same was done with the Schott flask used for the stock solution, beforehand). On the morning of the test, Petri dishes were emptied and refilled with 70 mL (pre-exposure) and three mL (test Petri dishes) solution. For spawning, Plexiglas boxes 20 x 20 x 6 cm in size and covered with a mesh grid to keep zebrafish from feeding on their own eggs were used as breeding boxes. They were topped with artificial sea grass acting as an optical spawning stimulus and were placed into the fish tanks the evening before the start of the test. Zebrafish spawn at sunrise; therefore, spawning in the laboratory starts with the onset of light the next morning. Eggs were collected with a sieve, rinsed with tepid tap water, transferred into pre-exposure Petri dishes and incubated for 2 h at 26 ± 1 °C. Following the pre-exposure, eggs for the test were chosen with regard to their age and developmental stage (0 hours post fertilization (hpf) ≙ 8 a.m.), placed into the small 30 mm Petri dishes and stored in a heated cabinet at 26 ± 1 °C. A total of 32 individuals were used per treatment, that is, four per Petri dish and eight replicates each. Embryos were checked every 12 to 24 h. Endpoints investigated under a stereo microscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) included mortality, developmental delays at 24 hpf, heart rate at 48 hpf, hatching success from 60 to 96 hpf and malformations at 96 hpf. Except for mortality, analysis of all endpoints, including hatching success, was based on living embryos/larvae at the respective time point of evaluation.

Table B.9.2.2-1: Overview of observed lethal and sublethal endpoints at respective time points.

Endpoint	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Mortality	✓	✓	✓	✓	✓	✓
Developmental delays		✓				
No somites		✓				
Non-detachment of the tail		✓				
No development of the eyes		✓				
Heart rate			✓			
Hatching success				✓	✓	✓
Malformations						✓
Oedema						✓
Eye/brain defects						✓
Deformation of the spine						✓
Light pigmentation						✓

Heart rates were determined from two out of four individuals per Petri dish for 20 s, and values were extrapolated to 1 min. Coagulated eggs, dead larvae and empty egg shells were removed from the Petri

dishes to avoid depletion of oxygen due to biological degradation processes. The embryo test was run three times and conducted according to Organisation for Economic Co-operation and Development (OECD) 236 (2013). The compound 3,4-dichloraniline (98%, CAS: 95-76-1; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at a concentration of four mg/L served as a positive control and reconstituted water, as a negative control. According to the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals for scientific purposes, D. rerio embryos and larvae that do not feed independently are not regarded as animals, thus regulations and permissions for animal testing do not apply. Nevertheless, all embryos in our tests were handled in the least stressful way possible and with the utmost care. After test termination embryos/larvae were euthanized with MS222.

Statistics; All statistical analyses were conducted in JMP 11.2.0 (SAS Institute Inc., Cary, NC, USA). Mortality, hatching success and the malformation rate at 96 hpf, as well as developmental delays at 24 hpf, were analysed with a likelihood-ratio χ^2 test, followed by Fisher's exact test. Finally, the sequential Bonferroni-Holm method was applied accounting for multiple testing. A Cox regression was used to assess mortality and hatching success over time. For the analysis of heart rate, the data were averaged per Petri dish and checked for a normal distribution and homogeneity of variances. Subsequently an ANOVA with Tukey's HSD or Dunnett's test was conducted. If data did not meet the criteria for an ANOVA and transformation of the data did not lead to the desired result, a non-parametric Steel-Dwass test was conducted instead. Additionally, for assessing the pH range in which pH control and glyphosate treatments differed in heart rate across the whole span of tested pH, non-linear regression analysis, including calculation of 95% confidence intervals (TableCurve 2D v5.01; SYSTAT Software Inc., San Jose, CA, USA), was applied. Non-linear regression analysis by TableCurve was also used for determining LC10/EC10 and LC50/EC50 values of endpoints in unbuffered glyphosate treatments.

Results

After 96 hpf, mortality and hatching success were 0% and above 80%, respectively, in control embryos. The 3,4-dichloraniline positive control induced high mortalities, with rates consistently above 80% after 96 hpf. Thus, the validity criteria according to Organisation for Economic Co-operation and Development (OECD) 236 (2013), including sensitivity of zebrafish, were met.

Unbuffered glyphosate: At the two highest concentrations tested (1 and 10 mM), it was already difficult to select well-developed eggs after the 2 h pre-exposure period. The yolk sac, which usually has a regular spherical shape, was found to be asymmetric and partly oval, and the chorion fluid, which is naturally clear, was murky in some cases and contained indefinable streaks.

As early as 12 hpf, all individuals, without exception, in the 10 mM treatment died. Mortality in the 1 mM exposure experiment was beyond 85% at 12 hpf and reached 100% within the first 24 h. Within the 750 µM glyphosate treatment, only six out of a total of 96 individuals survived until the end of the test at 96 hpf, whereas concentrations of 250 µM and below resulted in negligible or no mortality (3.125%). Regarding mortality at 96 hpf, all treatments 500 µM were highly significantly different from the control (likelihood ratio χ^2, $p < 0.001$). Lethal concentrations were calculated to be 385 µM (LC10) and 582 µM (LC50) at 96 hpf. Heart rates showed a concentration-dependent relationship, decreasing with increasing glyphosate concentration.

The mean heart rate was 149 beats per minute (bpm) for the control and between 130 and 140 bpm for low (10, 50 µM), 120 and 130 bpm for medium (100, 250 µM) and 110 and 120 bpm for the higher (500, 750 µM) concentrations. Thus, differences between the control and the 750 µM concentration ranged between 30 and 40 bpm. The treatments with the highest concentrations of glyphosate (1 mM, 10 mM) could not be evaluated due to 100% mortality at that time point. Only two individuals out of those exposed to 1 mM glyphosate survived until 60 hpf and seemed to continue the observed relationship between glyphosate and heart rate by showing even lower rates (93 and 96 bpm). As single individuals, they were not included in the statistical analysis. All remaining treatments were significantly different from the control (ANOVA with Tukey's HSD, $p < 0.001$) and the relationship between glyphosate concentration and heart rate could be described by linear regression analysis ($R2 = 0.546074$, $p < 0.001$). The EC10 was 43 µM.

Concerning the hatching rate, we observed a clear division between a cluster of treatments that comprised the control treatment and lower concentrations of glyphosate (10, 50, 100 µM) and another treatment cluster comprising higher concentrations (250, 500, 750 µM).

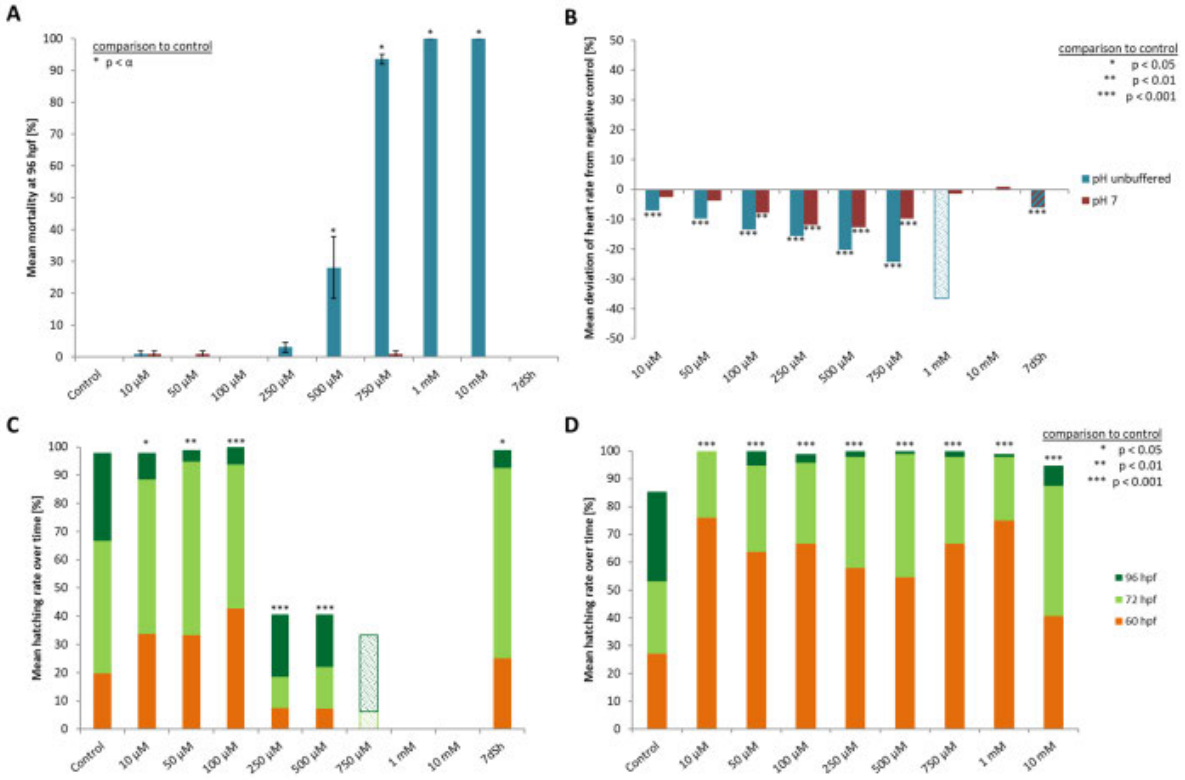


Figure B.9.2.2-1: Mortality, heart rate and hatching success in percentage of unbuffered and pH 7 treatment. (A) Mortality after 96 hpf (likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm, $p < \alpha$), (B) heart rate at 48 hpf relative to the negative control (Steel-Dwass, $p < 0.01$), (C) hatching rate over time in unbuffered treatments (Cox regression, $p < 0.05$), (D) hatching rate in pH 7 treatments over time (Cox regression, $p < 0.001$); shaded bars mark treatments with $n < 5$ that show tendencies but are not included in the statistical analyses.

Embryos exposed to lower concentrations hatched in 98–100% of cases, whereas hatching success in the experiments with 250 and 500 µM glyphosate was approximately 40%. All glyphosate treatments showed significant differences compared with the control (Cox regression, $p < 0.05$). EC10 and EC50 levels at 96 hpf were 155 and 224 µM, respectively.

There were no developmental delays at 24 hpf for glyphosate concentrations between 10 and 100 µM, whereas in treatments with 250 to 750 µM, rates varied from 15% to 25%. The EC10 for this endpoint was 126 µM. Results for all concentrations 250 µM were highly significant (likelihood ratio χ^2, $p < 0.001$) compared with the control. A direct concentration dependency could not be observed. Rather, it seemed that a distinct concentration threshold had to be exceeded to induce those developmental delays and failures, which later approached the same level. Prevalent defects were a lack of tail detachment, sometimes combined with apically curved tails; a lack of somite formation and an impairment of eye development was not detected. Occasionally, embryos were fully developed but either the complete tail or just the posterior end of their tails remained attached to the yolk sac. Under normal conditions, movement begins after tail detachment. Yet, even the embryos in glyphosate treatments that lacked tail detachment, overall development had progressed to a point at which muscular contractions were already visible. But due to the undetached tails, embryos were unable to turn around and their movement was very limited. Additionally, some embryos had the posterior end of their tails detached but displayed severe spine deformations. Those embryos could not move their tails in the same fluid manner as normally developed embryos could.

Malformations could be found in embryos of all glyphosate treatments but with rates below 20%. All glyphosate treatments were significantly different from the control. Among the malformations recorded, lightly pigmented embryos and larvae were particularly frequent). Furthermore, reduced eye size occurred regularly, and some individuals suffered from cardiac or yolk sac oedemas. Two individuals showed a notable shortening of the tail. Deformations of the spine at 96 hpf were observed surprisingly rarely, despite the high rates of tail and spine malformations at 24 hpf.

Table B.9.2.2-2: Results for concentration-dependent glyphosate treatments, as well as for pH-dependent control and glyphosate treatments, as percentages.

	Mortality		Hatching		HR	D	M
	96 hpf (%)	Over time	96 hpf (%)	Over time	48 hpf (bpm)	24 hpf (%)	96 hpf (%)
Unbuffered							
Neg. control	0	–	97.92	–	148.75	0	0.26
10 µM	1.04	n.s.	97.92	*	**138.38*** **	0	**2.36***
50 µM	0	n.s.	98.96	*	**134.19*** **	0	**5.47***
100 µM	0	n.s.	100	***	**128.69*** **	0	**4.69***
250 µM	3.13	n.s.	**40.65***	***	**125.56*** **	**22.23***	**13.57***
500 µM	**28.13***	*	**40.58***	***	**118.63*** **	**20.36***	**16.06***
750 µM	**93.64***	***	**33.33***	n.a.	**94.50*** **	**19.04***	**18.06***
1 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
10 mM	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
LC_{10}/EC_{10}	385 µM		155 µM		43 µM	126 µM	179 µM
LC_{50}/EC_{50}	582 µM		224 µM		–	–	–
7dSh	0	n.s.	98.93	*	**139.75*** **,†**	0.35	**0.27†**
Neutral (pH 7)							
Neg. control	0	–	85.42	–	148.04	0	0.52
10 µM	1.04	n.s.	**100***	***	144.25	0	1.04
50 µM	1.04	n.s.	**100***	***	142.56	1.04	1.87
100 µM	0	n.s.	**98.96***	***	**136.57****	0	2.60
250 µM	0	n.s.	**100***	***	**130.31*** **	0	0.26
500 µM	0	n.s.	**100***	***	**129.19*** **	0	1.56
750 µM	1.04	n.s.	**100***	***	**133.50***	0	2.35
1 mM	0	n.s.	**98.96***	***	145.94	0.69	**6.56***
10 mM	0	n.s.	**94.79***	***	149.38	0	**7.29***
pH range—control							
Neg. control	**0**	–	94.69	–	160.38	0	0.26
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**84.03***	***	**61.11***	***	**140.19*** **	**9.36***	**4.17***
pH 3.4	**51.04***	***	**77.78*,†**	***	**141.38*** **	**6.93***	**3.51***
pH 3.5	**8.33***	n.s.	**15.77*,†**	***,†	**144.25*** **	**8.32***	**2.0***
pH range—glyphosate							
pH 3	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.1	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.2	**100***	***	n.a.	n.a.	n.a.	n.a.	n.a.
pH 3.3	**72.57***	***	83.33	***	**136.46*** **	**13.33***	0
pH 3.4	**28.47***	***	**60.61*,†**	***	**141.49*** **	**4.54***	**3.80***
pH 3.5	**17.36***	n.s.	**66.57*,†**	***,†	**143.50*** **	**10.31***	**2.04***

Notes:
Asterisks (*) and bold indicate statistically significant differences from the negative control (Cox regression, ANOVA. $^{*}p < 0.05$. $^{**}p < 0.01$. $^{***}p < 0.001$. Likelihood ratio χ^2, Fisher's exact test, Bonferroni-Holm: $^{*}p < \alpha$).
Crosses (†) denote additional statistical significances between pH control and glyphosate within the same pH range or, in the case of 7dSh, differences from 1 mM glyphosate at pH 7. For unbuffered glyphosate concentrations, endpoint-related LC_{10}/EC_{10} and LC_{50}/EC_{50} values are given.
HR, heart rate; D, developmental delays; M, malformations; n.s, not significant; n.a., not available (no sufficient sample sizes for statistical analysis).

Glyphosate at pH 7; When the glyphosate solutions were adjusted to pH 7, almost no mortality or developmental delays occurred, and malformation rates were below 10% but were still significantly elevated in 1 and 10 mM treatments (likelihood ratio χ^2, $p < 0.001$). In the concentration range of 10 to 500 µM, heart rates showed a similar trend to those in unbuffered treatments but at a lower level: bpm decreased with increasing concentration. Still, treatments between 100 and 500 µM differed significantly from the negative control (Tukey's HSD, $p < 0.01$). At 750 µM, heart rates increased again, with a higher frequency than at 250 and 500 µM. At the two highest concentrations (1, 10 mM), heart

rates were, on the one hand, marginally decelerated (1 mM) and on the other hand, marginally accelerated (10 mM) compared with the negative control. Thus, it seems that there is a turning point between 500 and 750 µM, at which the relationship between increasing concentration and heart rate shifts from deceleration to acceleration in comparison with the negative control. As already seen for lower concentrations in unbuffered treatments, glyphosate tends to induce early hatching, even at the lowest concentration and independently of concentration. This effect unfolded to its true extent in the pH-neutral treatments. At least twice as much larvae had hatched across all glyphosate treatments at 60 hpf compared with the negative control. After 72 hpf, all larvae were hatched in glyphosate treatments, except for single individuals that hatched at 96 hpf or did not hatch at all, whereas in the negative control, only 53% of the embryos were hatched at 72 hpf and even about 15% remained unhatched at 96 hpf.

pH range , In a first step, 1 mM glyphosate was tested at pH 3, 4, 5, 6, 7 and 8 in comparison with negative controls at the respective pH but without the pesticide. Mortality was 100% for both treatments at pH 3, independent of the presence of glyphosate. Only a single individual survived the first 12 hpf. In contrast, only one individual died throughout all other exposures within 96 hpf. Morphological aberrations described for high glyphosate concentrations under unbuffered conditions also applied to low pH treatments, independent of glyphosate addition. Concerning sublethal endpoints, results between different acidities in the range of pH 4 to 8, as well as between control and glyphosate within the same pH range, were inconspicuous for the most part. Thus, the pH 3 to 8 series was tested just once, and subsequent testing concentrated on the range from pH 3 to 4. Thus, in the next step, pH 3, 3.25, 3.5, 3.75 and 4 were investigated in detail. As embryos exposed to pH 3.75 and 4 did not show any prominent effects, only a single run was conducted, and the final testing scheme was determined from pH 3 to 3.5 in 0.1 increments. Additionally, a test with unbuffered glyphosate at a one mM concentration (which resulted in a pH of 3.2 in the test solution) was included for direct comparison.

Mortality decreased with increasing pH. Treatments with a pH of 3.2 and lower induced 100% mortality after 96 hpf. Whereas embryos exposed to pH 3 and 3.1 died within 48 hpf at the latest, embryos in pH 3.2 treatments survived considerably longer.

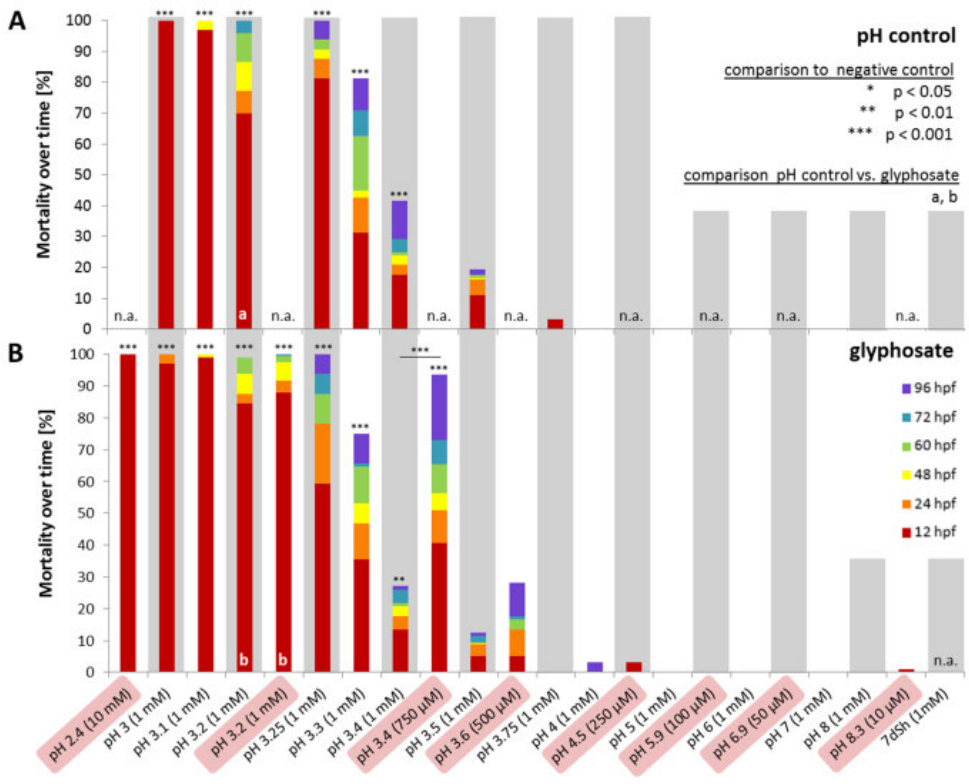


Figure B.9.2.2-2: Mortality over time as percentages of embryos exposed to the pH control (A) and glyphosate (B). Respective concentrations of glyphosate are given in brackets. Results from unbuffered treatments (50 µM–10 mM glyphosate; highlighted in red) are combined with pH range results and positioned according to their measured pH. Treatments not conducted in the pH control or glyphosate scheme are labelled n.a. (not available).

Significant differences from the negative control are marked with asterisks (*), except glyphosate pH 3.4 with an additional significant comparison between unbuffered and pH 7 treatment. Significances between pH control and glyphosate treatments within respective pH ranges are denoted with letters (a and b) (Cox regression, $p < 0.01$).

Apart from pH 3.5 without glyphosate, all treatments showed elevated mortality rates compared with the negative control (Cox regression, $p < 0.05$). There were no differences between control and glyphosate treatments with corresponding pH values, except for the elevated mortality in unbuffered glyphosate compared with the respective pH 3.2 control.

Compared with the negative control, hatching was significantly delayed and also reduced in both glyphosate and pH control treatments (Cox regression, $p < 0.001$). Whereas 30% of the control embryos hatched at 60 hpf, in the pH control and glyphosate exposures, the hatching rate at 60 hpf was consistently below 5% (see Supplementary File, hatching rate). The tendency toward glyphosate-induced premature hatching at 60 hpf that was observed in pH-neutral treatments was not evident at low pH. Although not statistically significant (except for pH 3.5: Cox regression, $p < 0.001$), embryos exposed to glyphosate tended to hatch earlier and more frequently than embryos in the respective pH controls.

Heart rates were significantly lowered by glyphosate at pH 3.3 to 3.5, as well as by the corresponding control pH treatments (Steel-Dwass, $p < 0.001$). Differences between glyphosate and the respective controls at the same pH value could only be detected when the full pH range dataset (including results for pH 3 to 8) was analyzed. At a pH between 5.55 and 6.02, glyphosate elevated the embryonic heart rate significantly compared with pH controls (TableCurve 2D v5.01). Developmental delays and malformations occurred in the low pH treatments, but they did not vary in a pH-dependent manner, and there was no detectable difference between glyphosate and the respective pH controls.

Comparison; When datasets for the unbuffered glyphosate treatment and the pH range were merged regarding mortality in relation to pH, interestingly, embryos exposed to unbuffered glyphosate showed higher mortalities at 500 and 750 µM compared with their 1 mM counterparts at pH 3.5 and 3.4, respectively. The unbuffered 750 mM treatment with a pH of 3.4, in particular, resulted in a mortality rate more than twice as high as that in the glyphosate pH 3.4 treatment (1 mM), mirroring mortality effects seen in treatments ranging rather between pH 3.25 and 3.3.

Conclusion
In unbuffered glyphosate medium the lethal concentrations were calculated to be 385 µM (LC_{10}) and 582 µM (LC_{50}) at 96 hpf. Regarding heart rates the EC_{10} was 43 µM. Concerning the hatching rate, EC_{10} and EC_{50} levels at 96 hpf were 155 and 224 µM, respectively. For developmental delays at 24 hpf the EC_{10} was 126 µM.

Assessment and conclusion by applicant:

For Zebrafish (*Danio rerio*) embryos acutely exposed to glyphosate at concentrations between 1.69 and 1690.7 mg glyphosate/L (10 µM to 10 mM) for 96 hours post fertilization (hpf) the LC_{10} and LC_{50} values (96 hpf) were calculated to be 65.1 mg a.s./L (385 µM) and 98.4 mg a.s./L (582 µM), respectively (in unbuffered glyphosate medium). Regarding heart rates the EC_{10} was 7.27 mg a.s./L (43 µM). Concerning hatching rate, 96 hpf -EC_{10} and EC_{50} values were 26.2 mg a.s./L (155 µM) and 37.9 (224 µM), respectively. For developmental delays at 24 hpf the EC10 was 21.3 mg a.s./L (126 µM). The test was conducted according to OECD 236 test guideline.

Concerning the validity criteria in the OECD 236, despite the stated > 80% mortality in the positive control (>30% required) there are no details presented to confirm the level of mortality. The fertilisation rate of the batch of eggs used was not reported. Finally, acute endpoints based on developmental delay and heart rate are not relevant to an EU level risk assessment for Annex I renewal purposes.

The test design is adequately described, however, there was no analytical verification of test concentrations reported. The study is considered as reliable with restrictions.

Assessment and conclusion by RMS:

The aim of this study was to differentiate the effects of glyphosate-induced acidification of the medium and those exerted by the compound itself (independent of low pH) on embryonic and early larval development of *Danio rerio*.

As highlighted above by the applicant, some validity criteria (of the OECD 236) are not reported: The fertilisation rate of the batch of eggs used was not reported. Despite the stated > 80% mortality in the positive control (>30% required) there are no details presented to confirm the level of mortality (note that toxic reference is not meant to be tested in each experiment but ideally twice a year in the laboratory).

Acute endpoints based on developmental delay and heart rate are not directly in the scope of EU risk assessment for Annex I renewal purposes. However a potential adverse effect on these parameters may indirectly represent an adverse effect on fish populations in natural conditions. Therefore RMS also reported the overall conclusions made on these parameters as they may be necessary for further investigations.
There was no analytical verification of test concentrations reported. RMS considers this study as reliable with restrictions and results are considered supportive.

For Zebrafish (*Danio rerio*) embryos acutely exposed to glyphosate at concentrations between 1.69 and 1690.7 mg glyphosate/L (10 µM to 10 mM) for 96 hours post fertilization (hpf):
The LC10 and LC50 values (96 hpf) were calculated to be 65.1 mg a.s./L (385 µM) and 98.4 mg a.s./L (582 µM), respectively in unbuffered glyphosate medium.
Regarding heart rates the EC10 was 7.27 mg a.s./L (43 µM).
Concerning hatching rate, 96 hpf -EC10 and EC50 values were 26.2 mg a.s./L (155 µM) and 37.9 (224 µM), respectively.
For developmental delays at 24 hpf the EC10 was 21.3 mg a.s./L (126 µM). Glyphosate significantly accelerated hatching compared with the control (all tested concentrations).
Malformations could be found in embryos of all glyphosate treatments but with rates below 20%. EC10 was 30.2 mg a.s./L (179 µM).

At test concentration 1 mM glyphosate (169.07 mg glyphosate/L), no mortality occurred, neither in the control nor in glyphosate treatments for pH greater than 4.

Globally the study demonstrates that the severe effects detected seemed to be mainly caused by a low (glyphosate induced) pH, the compound glyphosate itself affects embryonic development in *Danio rerio* at a sublethal level.

Data point:	CA 8.2.1
Report author	Gaur H. et al.
Report year	2019
Report title	Glyphosate induces toxicity and modulates calcium and NO signaling in zebrafish embryos.
Document Source	Biochemical and biophysical research communications (2019 Vol. 513, No. 4, pp. 1070
Guidelines followed in study	None
Deviations from current test guideline	Not applicable

GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Zebrafish embryos (5 hours post fertilization) were used to evaluate the toxic effects of glyphosate on the hatching rate and mortality and its mechanisms. Therefore, embryos were exposed to glyphosate concentrations of 10, 50, 100, 200, 400 µg/mL. All treatments were done as semi-static treatment with a daily renewal of the test medium in 24-well plate during 96 hours of exposure.
Glyphosate induced significant toxicity in a time and concentration-dependent manner. An LD_{50} of 66.04 ± 4.6 µg glyphosate/mL was determined after 48 h of exposure. Glyphosate significantly reduced the heartbeat in a time and concentration-dependent manner indicating cardiotoxicity. The results indicate that glyphosate induces significant toxicity including cardiotoxicity in zebrafish embryos in a time and concentration-dependent manner.

Materials and methods

Zebrafish housing and breeding
Adult zebrafish of mixed gender were purchased from a local commercial supplier. Fertilized eggs were produced, collected and stored in E3 medium (1X sterile E3 medium (in mM) as 9.92 NaCl, 0.35 KCl, 0.65 $CaCl_2 \cdot * 2H_2O$ and 0.8 $MgCl_2 \cdot * 6H_2O$: pH 7.2). The embryos were kept in the incubator at 28 ºC for 4-5h before chemical exposure.

Drug treatment
Effect of glyphosate (Sigma Cat # 45521) on the hatching rate and mortality of zebrafish embryo was assessed. After 5 hours of initial incubation, the embryos were exposed to various concentrations of glyphosate 10, 50, 100, 200, 400 µg/mL prepared in 1X E3 medium. All treatments were done as semi-static treatment in 24-well plate till 96 h at 28 ºC. Embryos 5 hpf (hours post fertilization) were incubated in respective glyphosate solutions, each well containing up to a maximum of 10-12 embryos and 400-500 µL of the respective glyphosate solution. Control embryos were maintained in the E3 medium. Glyphosate solutions were changed after every 24 hours and observations (including morphological abnormalities, hatching rate, mortality rate and eye malformations) were recorded.
LD_{50} was calculated from mortality data by plotting percent mortality against each concentration and fitting the data with a non-linear curve fitting using Hill's equation in Origin Pro 2018.

Imaging/video recording of the embryos
All the embryos were imaged by individually placing them on a glass slide or by directly placing the multi-well plates under the inverted bright field microscope, Olympus lX73 equipped with Procam HS-10 MP camera. The anterior length (AL) and posterior length (PL) of the eye were calculated from the captured images using software ImageJ.

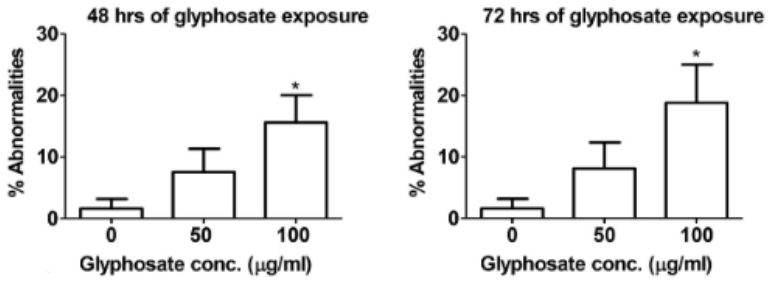
Statistical analysis
All data were analyzed using GraphPad Prism version 5 and Origin Pro 2018. All data are presented as mean ± SEM for the indicated n numbers. Paired or unpaired t-test was performed for parametric data and Mann-Whitney test for non-parametric data between two groups as applicable. P value of < 0.05 was considered significant, where * denotes $P \leq 0.05$; ** denotes $P \leq 0.005$ and *** denotes $P \leq 0.0005$ unless indicated otherwise.

Results

Glyphosate induced teratological lesions in zebrafish embryos

Zebrafish embryos treated with 50 and 100 µg/mL glyphosate showed abnormalities like pericardial edema, yolk sac edema and tail bending in the treated embryos, while control group larvae showed no such abnormalities. No morphological abnormalities in the embryos treated with 10 µg/mL glyphosate were observed. Percent abnormality was quantified in terms of total abnormalities which included pericardial edema, yolk sac edema and body malformations. A concentration and time dependent effect of glyphosate was observed on the morphology of zebrafish embryos. Glyphosate also induced a significant reduction in the anterior and posterior length of the eye at the higher concentration of 100 µg/mL glyphosate both after 48 and 72 h of glyphosate exposure. Further, reduction in the surface area of the eye was also observed after 72 h of glyphosate exposure in 50 and 100 µg/mL glyphosate concentrations.

Figure 1. Glyphosate induced morphological abnormalities in zebrafish embryos. Total % abnormalities (including any kind of abnormality) that were observed after 48 and 72 hours of glyphosate exposure. Data represents mean ± SEM of 12-54 individual zebrafish larvae for each treatment group. * denotes $P < 0.05$.



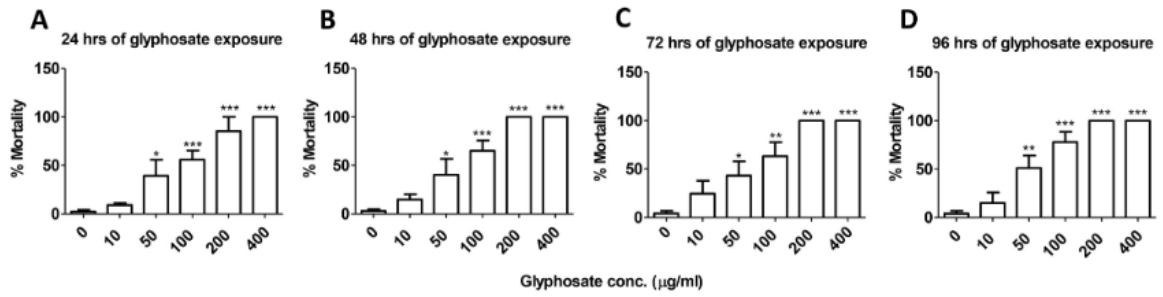
Glyphosate delayed hatching in zebrafish embryos

Hatching was significantly delayed in zebrafish embryos exposed to glyphosate at concentrations of 50 µg/mL and above.

Glyphosate affected the survival of zebrafish embryos

Cumulative mortality was recorded after 24, 48, 72 and 96 h of glyphosate exposure for zebrafish embryos exposed to various concentrations of glyphosate (10, 50, 100, 200 and 400 µg/mL). Mortality end-points included either coagulation of nuclear material or lack of heartbeat. A concentration and time-dependent effect of glyphosate on the mortality of zebrafish embryos was observed which was significant only at concentrations of 50 µg/mL and higher. At the highest concentration of 400 µg/mL glyphosate, 100% mortality was observed already after 24 h of glyphosate exposure. 200 µg/mL glyphosate induced 100% mortality after 48 h of glyphosate exposure. LD_{50} was calculated to be 66.04 ± 4.6 µg/mL after 48h of glyphosate exposure.

Figure 2. Glyphosate exposure induced mortality in zebrafish embryos. Mortality was recorded at four time points: 24 h, 48 h, 72 h and 96 h of glyphosate exposure. Percent mortality is plotted against indicated concentrations of glyphosate at 10, 50, 100, 200 and 400 µg/mL. A concentration and time-dependent effect of glyphosate is observed. Data represents mean ± SEM of 83-223 individual zebrafish larvae for each treatment group. * denotes $P < 0.05$, ** denotes $P < 0.001$ and ***denotes $P < 0.0001$.



Conclusion

The acute test with Zebrafish embryos (5 hours post fertilization) revealed a 48 hour-LD_{50} of 66.04 ± 4.6 µg glyphosate/mL.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: The approaches used are not EU-specific, thus the endpoints achieved cannot be used in an EU ecotoxicological regulatory risk assessment / glyphosate EU renewal. The study can only be considered supplementary (relevance Category B).

Assessment and conclusion by RMS:
Effect of glyphosate on the hatching rate and mortality of zebrafish embryo was assessed. Observations including morphological abnormalities, hatching rate, mortality rate and eye malformations) were recorded. Embryos were exposed to glyphosate concentrations of 10, 50, 100, 200, 400 mg/L during 96 hours.
Zebrafish embryos treated with 50 and 100 mg/L glyphosate showed abnormalities like pericardial edema, yolk sac edema and tail bending in the treated embryos.
Hatching was significantly delayed in zebrafish embryos exposed to glyphosate at concentrations of 50 mg/mL and above.
These concentrations are above those expected under realistic conditions.
Mortality of zebrafish embryos was observed which was significant only at concentrations of 50 mg/mL and higher. LD50 was calculated to be 66.04 ± 4.6 mg/L after 48 h of glyphosate exposure.

Glyphosate significantly reduced the heartbeat in a time and concentration-dependent manner indicating cardiotoxicity. Selective downregulation of Cacana1C (L-type calcium channel) and ryr2a (Ryanodine receptor) genes along with selective upregulation of hspb11 (heat shock protein) gene was observed upon exposure to glyphosate indicating alterations in the calcium signaling. A reduction in the nitric oxide (NO) generation was also observed in the zebrafish embryos upon exposure to glyphosate.
The relevance of these results for the risk assessment cannot be established by RMS as no quantitative link can be made between observations on these parameters and the potential adverse at population level (this latter being the specific protection goal). The results on these parameters are not relevant for the risk assessment. The corresponding part of the summary is therefore not reported.

Only results on mortality and other sublethal effects were considered in deep by RMS that considers that they can provide further evidence for acute toxicity of glyphosate towards embryos of zebrafish.
No analytical verification is reported in this article.
RMS considers this study is relevant and reliable with restrictions.

Data point:	CA 8.2.2, CA 8.2.3, CP 10.2.2, CP 10.2.3
Report author	Uren Webster T. M. et al.
Report year	2014
Report title	Effects of glyphosate and its formulation, Roundup, on reproduction in zebrafish (*Danio rerio*).
Document Source	Environmental science & technology (2014), Vol. 48, No. 2, pp. 1271 - 1279
Guidelines followed in study	The study partly followed OECD guideline 229 (2009) (chemical exposure was done according to guideline)
Deviations from current test guideline	No (only applicable to exposure part)
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

The study aimed to investigate the reproductive effects of Roundup and glyphosate in fish and the potential associated mechanisms of toxicity. A 21-day exposure of breeding zebrafish (*Danio rerio*) was conducted to 0.01, 0.5, and 10 mg/L (glyphosate acid equivalent) Roundup and 10 mg/L glyphosate. 10 mg/L glyphosate reduced egg production but not fertilization rate in breeding colonies. Both 10 mg/L Roundup and glyphosate increased early stage embryo mortalities and premature hatching. However, exposure during embryogenesis alone did not increase embryo mortality, suggesting that this effect was caused primarily by exposure during gametogenesis. Transcript profiling of the gonads revealed 10 mg/L Roundup and glyphosate induced changes in the expression of cyp19a1 and esr1 in the ovary and hsd3b2, cat, and sod1 in the testis. The results demonstrate that these chemicals cause reproductive toxicity in zebrafish, although only at high concentrations unlikely to occur in the environment, and likely mechanisms of toxicity include disruption of the steroidogenic biosynthesis pathway and oxidative stress.

Materials and Methods

Fish Maintenance
Colonies of 4 male and 4 female adult (20 weeks old) WIK strain zebrafish were established in individual 15 L glass tanks and allowed to breed naturally during a 7 day acclimation period. Each tank was aerated and supplied with a water flow rate of 48 L /day. The aquarium water supply was reverse-osmosis treated tap water reconstituted with analytical-grade salts to produce standardized synthetic freshwater according to OECD guidelines, and maintained at 28 ± 0.5 °C and pH 7-7.5. Fish were kept under a 12h light:dark cycle and fed twice daily with live *Artemia nauplii* and flake food to satiation.

Chemical Exposures
Chemical exposure was conducted via a flow through system for a period of 21 days in accordance with OECD guidelines for fish reproductive tests, preceded by a 10 day pre-exposure period. The treatment groups consisted of three concentrations of Roundup; 0.01, 0.5, and 10 mg/L glyphosate acid equivalent (using Roundup GC liquid glyphosate concentrate containing 120 g/L glyphosate acid, Monsanto, Cambridge, U.K.), 10 mg/L glyphosate (analytical grade; Molekula, Wimborne, U.K.), and a control group. Each treatment group was comprised of three replicate breeding colonies (4 males and 4 females) in 15 L tanks. Water samples were collected from each tank on days 7, 14, and 21 of the exposure period and stored at -20 °C prior to chemical analysis. Glyphosate quantification was carried out using a 6420B Triple Quadrupole (QQQ) mass spectrometer coupled to a 1200 series Rapid Resolution HPLC system.

Reproductive Test and Embryo Exposures
Group spawning occurred daily at dawn and eggs were collected 1 h post fertilization (hpf), rinsed thoroughly to remove detritus and incubated in water containing the same chemical exposure concentrations as their tank of origin, at 28 °C. Exposure water for the embryo experiments was made according to the ISO 7346-3:1996 guidelines, fully oxygenated and supplemented with 2.5 µL/L of the antifungal agent methylene blue to avoid mortalities caused by fungal infections. The eggs from each colony were examined using light microscopy between 2 1/2 and 3 1/2 h after dawn, when all fertilized eggs had reached at least the 16-cell stage during early cleavage, and the total number of fertilized and unfertilized eggs were quantified on each day throughout the pre-exposure and exposure periods. During the 21-day chemical exposure, fertilized eggs displaying cellular necrosis were counted and recorded as early stage mortalities (<3.5 hpf). Fifty fertilized eggs from each tank were selected randomly and incubated in 50 mL exposure water until 72 hpf. During this period, embryo mortality was recorded at

24, 54, and 72 hpf and embryo hatching was recorded at 54 and 72 hpf. To determine if the observed effects of Roundup and glyphosate on embryos were because of the effects of exposure during gametogenesis or during embryogenesis, embryos collected from a control population were exposed to a range of concentrations of glyphosate and Roundup as above. Chemical treatment was initiated between 10 and 20 min post fertilization. In addition to the exposure concentrations used for the adult exposures, embryos were also treated with higher concentrations (50, 100, 250, 500, and 1000 mg/L a.e. Roundup and glyphosate) to determine the concentration thresholds for embryo mortalities and developmental toxicity. Experiments were conducted in triplicate; each replicate contained 50 embryos and observations of mortalities and hatching were performed as described above.

Sampling
All fish were humanely sacrificed on day 21 of the exposure period by a lethal dose of benzocaine (0.5 g/L) followed by destruction of the brain. Wet weight and fork length were recorded and the condition factor (k = (weight (g) × 100)/(fork length $(cm)^3$)) was calculated for individual fish. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/total weight (mg)) x 100)) was determined for individual fish. Gonads were dissected, weighed and one gonad from each fish was snap frozen in liquid nitrogen and stored at -80 °C prior to transcript profiling. The remaining gonad was fixed in Bouin's solution (Sigma-Aldrich) for histological analysis. The gonadosomatic index (GSI; gonad weight (mg)/total weight (mg)) × 100)) was determined for both males and females.

Transcript Profiling and Histological Analysis
Transcript profiling of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes, was conducted using RT-QPCR in the gonads of exposed fish. Histological analysis of the gonads was also conducted. Transcripts of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes were quantified in the gonads of exposed fish using real time quantitative PCR (RT-QPCR). RNA was extracted from the gonads of eight male and eight female fish from each treatment group using TRI reagent (Sigma Aldrich) according to the manufacturer's instructions. RNA concentration and purity were assessed with a NanoDrop ND-1000 Spectrophotometer. Histological analysis was conducted with a light microscope connected to an Olympus DP70 camera and using analySIS image processing 3.2 software. Two slides, each containing multiple sections, were prepared from portions of ovary 100 μm apart, to provide a representative analysis of the ovarian tissue, while single slides were prepared for each testis.

Statistical Analysis
Statistical analyses were conducted with SigmaStat (version 12.0). Before analysis, proportional data (embryo survival and hatching) were subjected to variance-stabilizing square-root or arcsine transformations as appropriate. All reproductive output and sampling data met assumptions of normality and equal variance. Outliers in transcript expression data were identified and removed according to Chauvenet's criterion prior to statistical analysis. Transcript expression data that did not meet normally distributed criteria was log transformed before statistical analysis. All data was analyzed using single factor one way analysis of variance (ANOVA), followed by the Holm-Sidak post hoc test using a pairwise comparison method. Data were considered to be significant when $P < 0.05$.

Results & Conclusion

Water Chemistry
The mean measured concentrations of glyphosate in the tank water were between 88% and 140% of the nominal values for all treatments (quantification of glyphosate in tanks receiving 0.01 mg/L Roundup was below the detection limit of our method) (Figure 1).

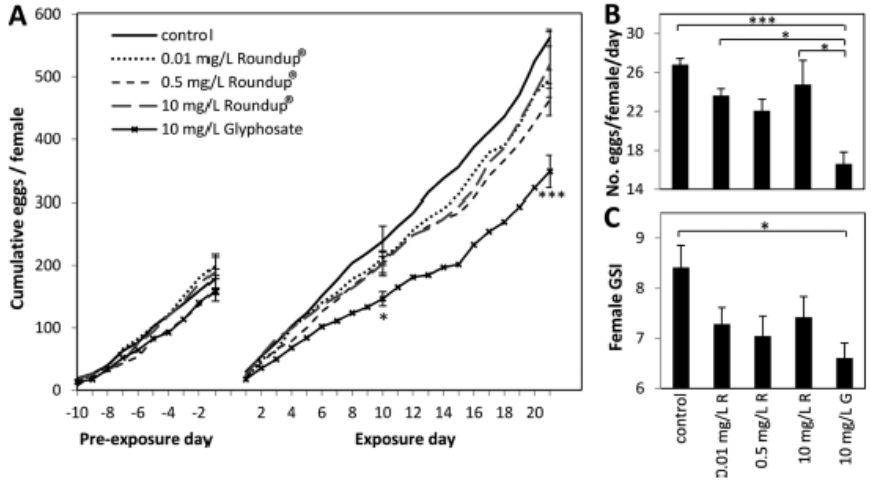
Figure 1. Water chemistry analysis of glyphosate in the exposure tank water. Data presented are the measured concentrations for the three replicate treatment tanks on days 7, 14 and 21 are presented as mean values ± SEM. Analysis was conducted using a 6420B Triple Quadrupole (QQQ) mass spectrometer coupled to a 1200 series Rapid Resolution HPLC system.

Nominal concentration	control	0.01 mg/L Roundup	0.5 mg/L Roundup	10 mg/L Roundup	10 mg/L glyphosate
Day 7	< 0.05	< 0.05	0.43 ± 0.1	8.8 ± 4.2	14.2 ± 1.9
Day 14	< 0.05	< 0.05	0.40 ± 0.1	13.3 ± 0.6	10.0 ± 0.6
Day 21	< 0.05	< 0.05	0.50 ± 0.1	16.3 ± 2.0	17.7 ± 1.5
Mean	**< 0.05**	**< 0.05**	**0.44**	**12.8**	**13.9**

Morphometric Parameters

The mean mass and length of male and female fish were 375.0 ± 6.3 mg/32.6 ± 0.2 mm and 402.6 ± 9.3 mg/31.7 ± 0.2 mm, respectively. There were no significant differences in size or condition factor (mean 1.08 and 1.25 for males and females, respectively) between treatment groups. Additionally, no alteration of general health or behavior in any colony was observed. The GSI of females was significantly lower in the fish treated with 10 mg/L glyphosate compared to the control group (Figure 2c). There was no significant difference in the GSI of males between treatment groups, or in the HSI of males or females.

Figure 2. (A) Cumulative egg production during the 10 day pre-exposure and 21 day chemical exposure periods (n = 3 replicate colonies per treatment). (B) Mean number of eggs laid per female per day throughout the 21 day exposure period (n = 3 replicate colonies per treatment), and (C) mean gonad-somatic index of females in each treatment group (n = 12 individual females per treatment). Data plotted are mean values ± SEM. Asterisks indicate significant differences between treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001).



Reproductive Test and Embryo Exposures

During the 10 day pre-exposure period, there was no difference in cumulative egg production between the treatment groups (P = 0.468). During the exposure period, colonies in the control group consistently spawned the greatest number of eggs per female, while those treated with 10 mg/L glyphosate spawned the least. From day 10 of the exposure period, cumulative egg production was significantly reduced in colonies exposed to 10 mg/L glyphosate compared to the controls, and this difference intensified throughout the remainder of the exposure period. At the end of the 21 day exposure, cumulative egg production was significantly lower in colonies exposed to 10 mg/L glyphosate compared to the control, and also compared to the 10 and 0.01 mg/L Roundup groups (Figure 2a,b). Additionally, egg output significantly correlated (R^2 = 0.79; P = 0.043) with female GSI across all treatment groups. Fertilization rate remained consistently high throughout the exposure period with no significant differences between treatment groups and an overall mean value of 83.4%. There was a significant increase in embryo mortalities occurring before 3.5 hpf in embryos from both the 10 mg/L Roundup and glyphosate treatment groups (Figure 3a). Additionally, there was a significant correlation between early embryo mortality and the concentration of Roundup (R^2 = 0.52; P = 0.008). There were no significant differences between treatments in embryo mortality between the start of epiboly (3.5 hpf) and the end of somitogenesis at 24 hpf (Figure 3b). However, there was a significant increase in the percentage of embryos that had hatched at 54 hpf in groups treated with 10 mg/L Roundup and 10 mg/L glyphosate compared to the control group (Figure 3b). For embryos originating from a control population, exposure to glyphosate and Roundup at the concentrations used in the adult reproductive test (0, 0.01, 0.5, and 10

mg/L Roundup and 10 mg/L glyphosate) did not result in increased mortality rate at either 3.5 hpf or 24 hpf (Figure 3a,b), but there was a significant increase in 3.5-24 hpf mortality in embryos exposed to concentrations ≥100 mg/L glyphosate and ≥500 mg/L Roundup (Figure 4a). Evidence was also observed of developmental delay and abnormalities from concentrations ≥50 mg/L glyphosate and ≥250 mg/L Roundup at 24 hpf. There was a trend toward increased hatching at 54 hpf in groups exposed to 10 and 50 mg/L Roundup and glyphosate, and there was a significant correlation between hatching rate at 54 hpf and exposure concentration of Roundup up to 50 mg/L (R^2 = 0.27; P = 0.04) (Figure 4b). For embryos exposed to ≥100 mg/L Roundup and glyphosate, we found evidence of progressive delay in development and hatching with increasing concentration.

Figure 3. Effects of Roundup and glyphosate on embryo survival and development. Black bars represent embryos originating from exposed parental populations (n = 3 replicate colonies, for each colony data was collected every day for 21 days of exposure and averaged) and gray bars represent embryos originating from a control parental population (n = 3 replicate exposures, each replicate containing 50 embryos). (A) Percentage of embryo mortalities that occurred before 3.5 hpf. (B) Percentage of embryo mortalities that occurred between 3.5 and 24 hpf. (C) Percentage of embryos that had hatched at 54 hpf in each treatment group. Data plotted are mean values ± SEM. Asterisks represent significant differences from the control treatment (***P < 0.001).

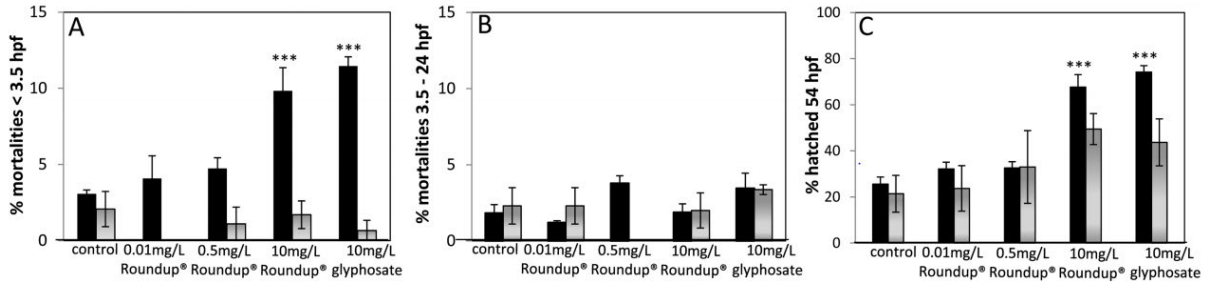
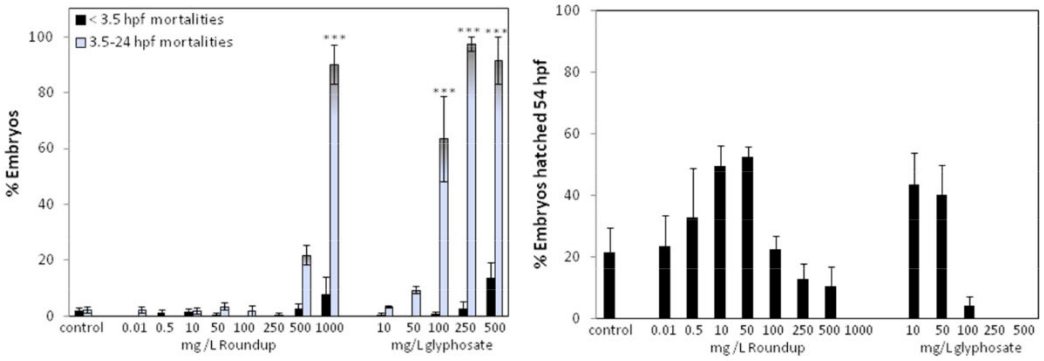


Figure 4. Effects of Roundup and glyphosate on the survival and development of embryos originating from the unexposed control parental population; **(A)** percentage of embryo mortalities that occurred before 3.5 hpf and between 3.5-24 hpf; and **(B)** percentage of embryos that had hatched at 54 hpf. Treatment concentrations include those used during the adult exposure, and higher concentrations (> 10 mg/L) to investigate the thresholds for mortality and development abnormalities to occur. Data presented are mean values ± SEM (n = 3 replicates per treatment concentration, each replicate consisted of 50 embryos). Asterisks indicate significant difference from the control group (*P<0.05 **P<0.01 ***P<0.001).



Gonad Transcript Profiling

In the ovary, the transcript encoding aromatase (cyp19a1) was significantly up-regulated in the 10 mg/L Roundup treatment group compared to the controls. Estrogen receptor 1 (esr1) in the 10 mg/L Roundup group was significantly up-regulated compared to the 10 mg/L glyphosate group. There were similar, but not statistically significant, decreasing trends in expression of other steroidogenic enzymes including cytochrome P450, subfamilies 17 and 11 *(cyp17a1*, *cyp11a1*) and 3β-hydroxysteroid dehydrogenase (*hsd3b2*) in groups exposed to both Roundup and glyphosate. In contrast, for the antioxidants glutathione peroxidase (*gpx1a*), catalase (*cat*) and glutathione-S-transferase pi (*gstp1*) non-significant, increasing trends in transcript expression were observed. In the testis, *hsd3b2* was significantly up-regulated following exposure to 10 mg Roundup/L compared to all other treatment groups. The expression pattern of steroidogenic acute regulatory protein (*star*), *cyp17a1*, *cyp11a1*, and the androgen receptor (*ar*)

additionally appeared to follow an expression pattern similar to *hsd3b2* across treatment groups. *cat* was significantly up-regulated in groups exposed to both 10 mg/L Roundup and 10 mg/L glyphosate compared to those treated with 0.5 mg/L Roundup. In addition, *sod1* was significantly upregulated in the 10 mg/L compared to 0.5 mg/L Roundup groups.

Table S1: Target genes, primer sequences and assay details for RT-QPCR analysis.

Gene Name	Gene Symbol	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)	Ta (°C)	PCR efficiency
Ribosomal protein L8	*rpl8*	CCGAGACCAAGAAATCCAGAG	CCAGCAACAACACCAACAAC	91	59.5	1.95
Catalase	*cat*	AGTTCCCTCTGATTCCTGTG	ATGGCGATGTGTGTCTGG	173	61.0	2.00
Superoxide dismutase	*sod1*	TTCACTCTCTCACAACTTCTC	GTCACCTTCACTGGCTTC	142	58.0	2.18
Glutathione peroxidase	*gpx1a*	CTGCGTGTTGCCCTTTGAG	GGTGTAATCCCTGACTGTTGTG	189	58.5	1.98
Glutathione S transferase pi	*gstp1*	AACGACAGTGAGGCTTCC	GCATTTGAGGTGGTTGGG	141	56.0	1.85
Glutathione S transferase alpha	*gsta1*	GGTGGCTCTTGGCTGTTG	TGCGATGTAGTTCAGGATGG	170	61.0	2.03
Steroidogenic acute regulatory protein	*star*	TTCTTGAGGACCAGGATG	GACTTGCTTGACATTGGG	197	58.0	2.03
Cytochrome P450, subfamily XIA, polypeptide 1	*cyp11a1*	TGAGTGCTGTGTTGTATG	AAATGTTGGACCCTATGG	159	57.0	2.12
Aromatase	*cyp19a1a*	AGCCGTCCAGCCTCAG	ATCCAAAAGCAGAAGCAGTAG	101	61.5	2.06
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	*hsd3b2*	GCAGCATTGAGGTAGCGTGTC	AGGATAAGAGGAGTAAGGCGTGTC	83	60.0	2.12
Cytochrome P450, subfamily XVIIA, polypeptide 1	*cyp17a1*	CGACAGTAAGATTGGGAAAGAAAG	GATGAGGAGCGGAGAAACAG	118	60.5	1.98
Estrogen receptor 1	*esr1a*	TATGACCTGTTGCTGGAGATG	CGCCGTTGGACTGAATGG	130	59.5	2.14
Estrogen receptor 2a	*esr2a*	AGGAGAAAACCAAGTAAACCAATC	AGGCTGCTAACAAGGCTAATG	173	59.0	1.86
Estrogen receptor 2b	*esr2b*	ATCTGCTAATGCTGCTCTCAC	CGCTCTGTTGTCTGTCTTCC	131	57.8	2.18
Androgen receptor	*ar*	ACGAGGGTGTTAGATGAGAC	AAGTATGAGGAAAGCGAGTAAAG	129	58.0	1.97
bcl2-associated X protein a	*baxa*	CTACTTTGCCTGTCGCCTTG	GTCCCATCCACCCTGTTCC	136	60.0	2.14
Tumour protein p53	*tp53*	GCTTGGTGCTGAATGGAC	GAGTGATGATTGTGAGGATGG	98	56.0	2.09

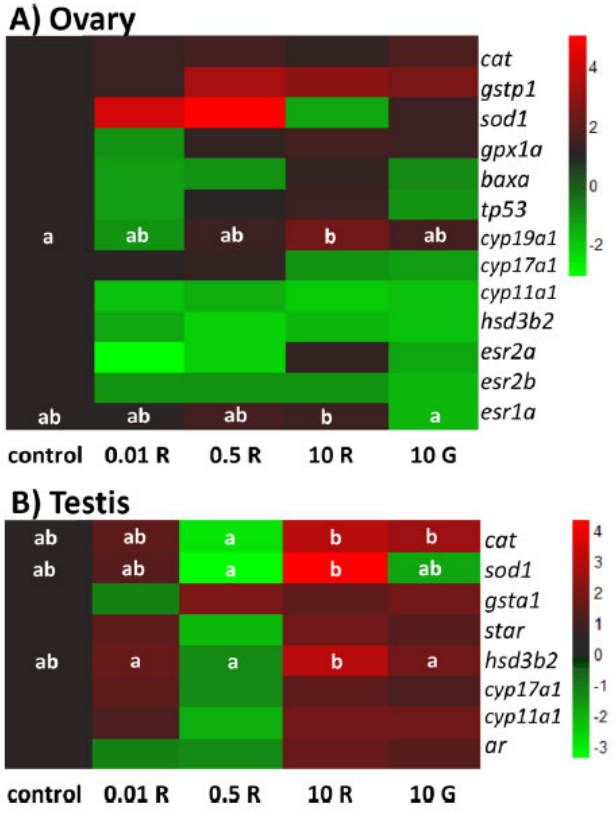


Figure 3. Transcript profiling of target genes in the ovary (A) and testis (B) following exposure to Roundup (R) and glyphosate (G). Data are presented as fold change relative to expression in the control group, whereby red shading indicates up-regulation and green shading represents down-regulation. Relative expression was calculated as ratio of target gene/rpl8 mRNA concentration. For each treatment, n = 6−8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the

analysis. Lettering indicates significant differences between treatment group, with groups identified with different letters being significantly different from each other ($P < 0.05$).

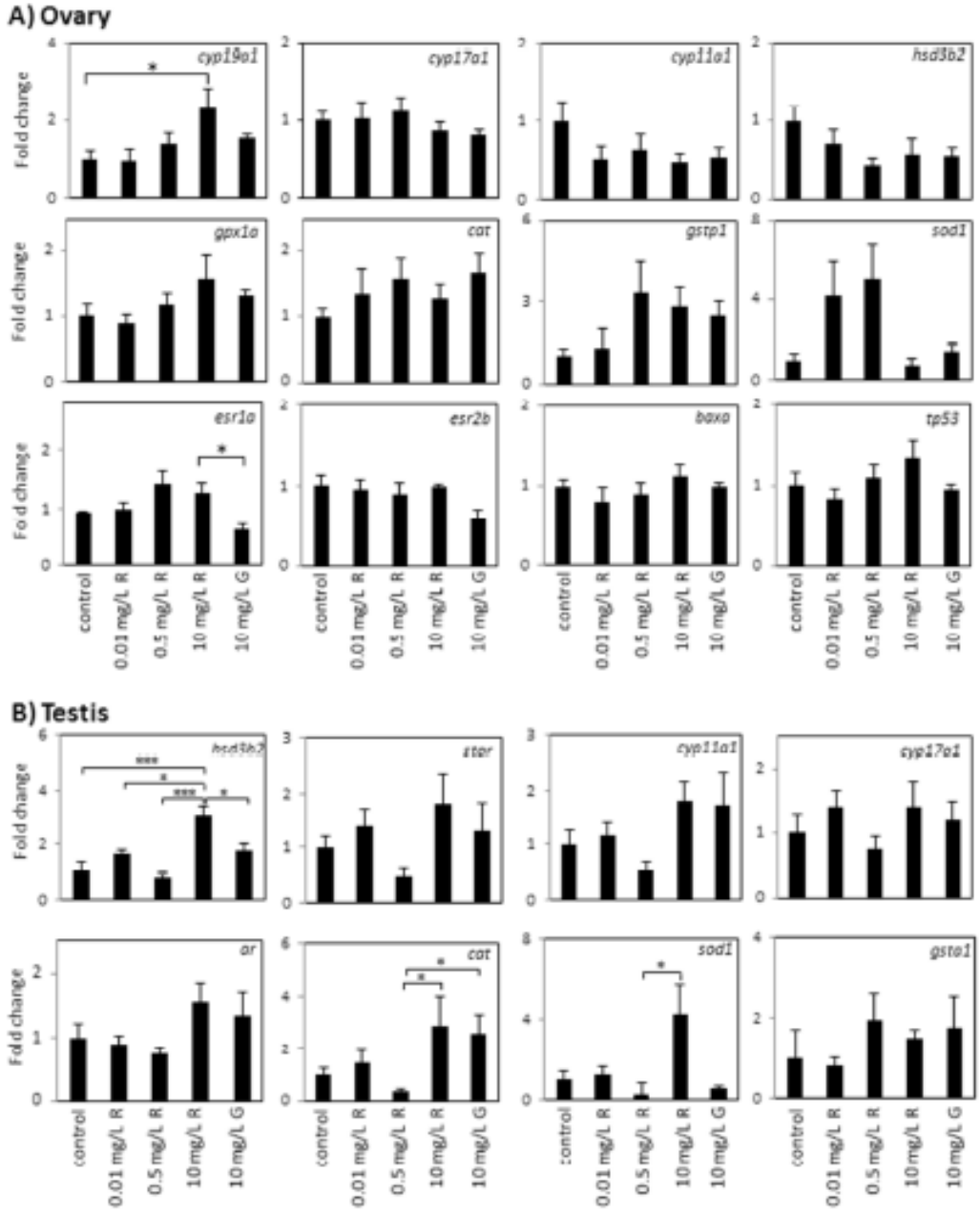


Figure S1. Transcript profiling of target genes in the ovary (A) and testis (B). Data are presented as fold change relative to expression in the control group. Relative expression was calculated as ratio of target gene /rpl8 mRNA concentration. For each treatment, data was collected for 6–8 fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis. Asterisks represent significant differences between treatment groups (*P<0.05 **P<0.01 ***P<0.001).

Gonad Histology

Histological examination of females from all treatment groups showed that the ovaries of all individuals contained oocytes at all stages of development (oogonia, primary oocytes, cortical alveoli stage oocytes, secondary oocytes, and mature vitellogenic oocytes) and the majority contained recent postovulatory follicles. Evidence was found of ovarian abnormalities in 9.1%, 18.2%, 9.1%, 50.0%, and 63.6% of females in the control, 0.01 mg/L Roundup, 0.5 mg/L Roundup, 10 mg/L Roundup and 10 mg/L glyphosate treatment groups, respectively. The majority of abnormalities were relatively mild and included accumulation of eosinophilic fluid and presence of abnormal tissue. In addition, the proportion of fish containing atretic oocytes in their ovaries also appeared to be increased (Figure 8). Histological

examination of males showed that testes of all individuals from all treatment groups contained germ cells at all stages of spermatogenesis (including spermatogonia, spermatocytes, spermatids, and mature spermatozoa). There were no abnormalities and no differences between stages of development between treatment groups.

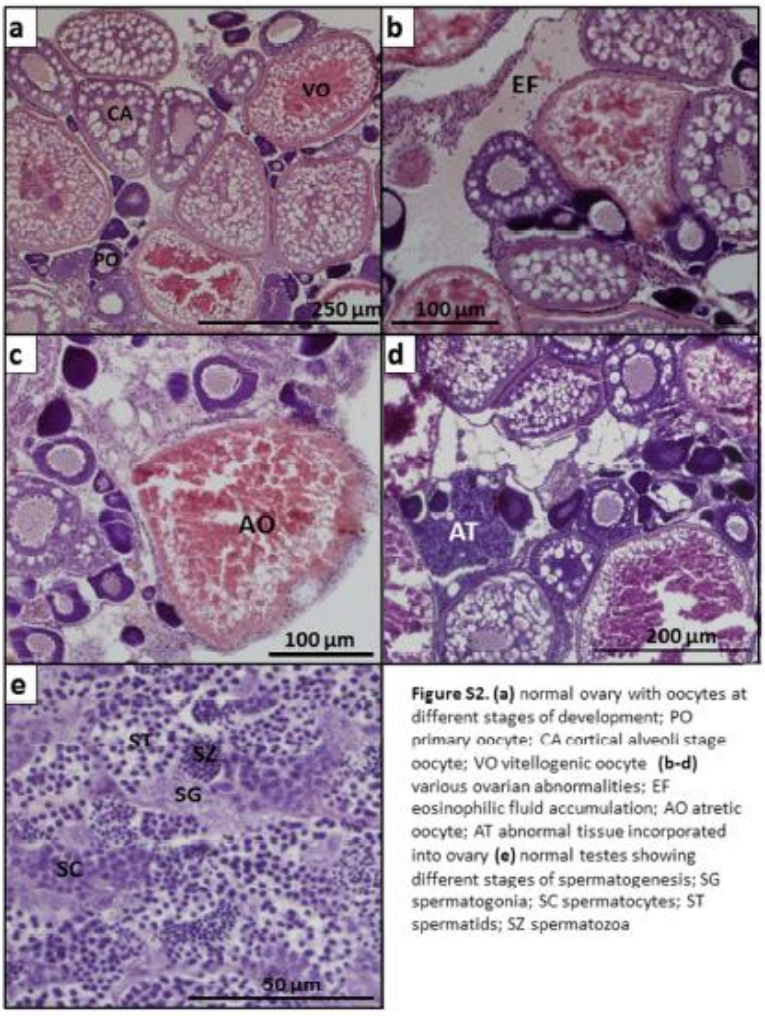


Figure S2. (a) normal ovary with oocytes at different stages of development; PO primary oocyte; CA cortical alveoli stage oocyte; VO vitellogenic oocyte **(b-d)** various ovarian abnormalities; EF eosinophilic fluid accumulation; AO atretic oocyte; AT abnormal tissue incorporated into ovary **(e)** normal testes showing different stages of spermatogenesis; SG spermatogonia; SC spermatocytes; ST spermatids; SZ spermatozoa

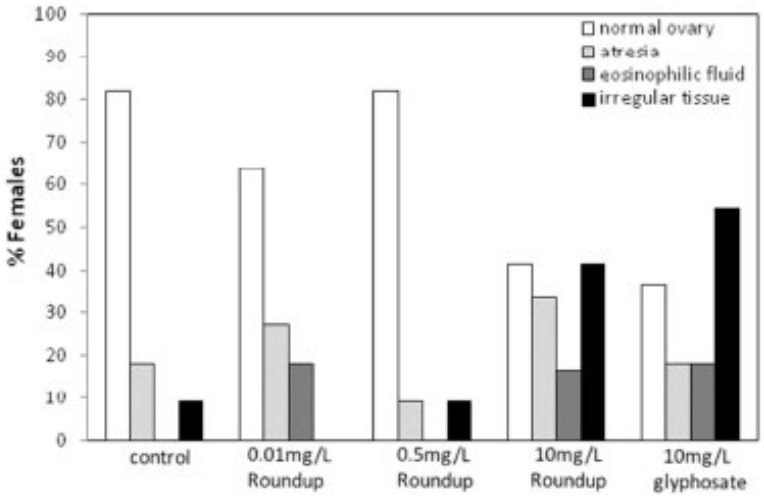


Figure S3. Histological analysis of the ovaries of females exposed to glyphosate and Roundup. Proportion of females in each treatment group showing absence or presence ovarian abnormalities (n=11-12 fish per treatment).

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The test substance Roundup GC is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). There was only a single glyphosate exposure group at 10 mg/L prepared from analytical grade. The purity of the material was not confirmed, but it was stated to be analytical grade. The study provides no endpoints for the glyphosate EU renewal, that could be used in the ecotoxicological regulatory risk assessment.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Roundup GC liquid was used but its co-formulants were not stated. The corresponding results are then considered less relevant but supplementary (and not considered thereafter).

Glyphosate was also used but there was only one single exposure group at 10 mg/L. Nevertheless this study investigates parameters of interest for the risk assessment.

This study states that (for glyphosate):

- 10 mg/L glyphosate reduced egg production but not fertilization rate in breeding colonies.

- 10 mg/L glyphosate increased early stage embryo mortalities and premature hatching. However, exposure during embryogenesis alone did not increase embryo mortality, suggesting that this effect was caused primarily by exposure during gametogenesis.

- 10 mg/L glyphosate induced changes in the expression of cyp19a1 and esr1 in the ovary and hsd3b2, cat, and sod1 in the testis. Trend of increase in the expression of ovarian aromatase, an enzyme which catalyzes the conversion of testosterone to estradiol in granulosa cells, in the gonads of females exposed to 10 mg/L glyphosate (which was significant for 10 mg/L Roundup). Likely mechanisms of toxicity include disruption of the steroidogenic biosynthesis pathway and oxidative stress. The relevance of these parameters for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level, however they are relevant for the assessment of the potential for endocrine effect.

LOEC reproduction =10 mg/L (only concentration tested).
No NOEC could be determined, then this study provides no endpoint usable for the risk assessment. However, it can be used in a Weight of evidence approach and the study is retained by RMS. RMS notes that this concentration is above concentrations measured to date in the environment and above the current endpoint on which the RAC is based on.

Reproductive effects may occur via multiple mechanisms of toxicity that may include disruption of the steroidogenic pathway and sex steroid signaling, and generation of oxidative stress. RMS notes that, as effects were observed, the concentration tested was likely above the MTC.
The study authors claim that early stage mortality was not the result of direct toxicity of the chemical exposure on embryos. Their assumption is based on the fact that exposed embryos originating from a control population of untreated adults exposed at concentrations of up to 10 mg/L of Roundup and 10 mg/L glyphosate had no effect on embryo survival at <3.5 or 3.5−24 hpf.
However RMS notes that the chosen glyphosate concentration of 10 mg/L is clearly above the NOEC based on mortality on zebrafish of 1 mg/L (Dias Correa Tavares, C.M., 2000 where mortality was of 26.7% at the tested concentration (nominal) of 10 mg/L).

The study is then of low relevance for the investigation of potential for endocrine disruption as effects seen above expected MTC.

This study is relevant and reliable (for toxic effects of glyphosate). The study is not reliable enough for its use in assessment of ED.

Data point:	CA 8.2.1
Report author	Zhang S. *et al.*
Report year	2017
Report title	Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*).

Document Source	Chemosphere (2017), Vol. 181, pp. 270-280
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

In this study, the authors employed larval zebrafish as an animal model to evaluate the effect of different concentrations of glyphosate on early development via morphological, biomechanics, behavioral and physiological analyses. Morphological results showed that an obvious delay occurred in the epiboly process and body length, eye and head area were reduced at concentrations higher than 10 mg/L. The expression of *ntl* (*no tail*) shortened and *krox20* (also known as *Egr2b, early growth response 2b*) changed as the glyphosate concentration increased, but there was no change in the expression of *shh* (*sonic hedgehog*). In addition, biomechanical analysis of the elasticity of chorion indicated that treated embryos' surface tension was declined. Furthermore, a 48-h locomotion test revealed that embryonic exposure to glyphosate significantly elevated locomotor activities, which is probably attributed to motoneuronal damage. The decreased surface tension of chorion and the increased locomotive activities may contribute to the hatching rates after glyphosate treatment.

Materials and Methods

Zebra fish maintenance
The zebrafish (AB strain) were raised at a constant temperature of 28.5 °C on a constant light cycle (14 h light/10 h dark). The fishfarming system was equipped with circulating water to maintain a standard water system (KCl 0.05 g/L, $NaHCO_3$ 0.025 g/L, NaCl 3.5 g/ L, and $CaCl_2$ 0.1 g/L, with 1 μg/mL methylene blue, pH 7.0 - 7.3). The zebrafish were fed fresh brine shrimp twice every day. In the night before experiment, one male and one female zebrafish were transferred to a breeding tank with a sliding door. The next morning, the sliding door was removed to allow the male to pursue the female. Sufficient eggs were collected in about 15 min after fertilization. The eggs were washed to remove the unfertilized eggs and debris. The normal fertilized eggs were used for subsequent experiments.

Experimental design and chemical exposure
The authors conducted a series of experiments to detect biological effects of glyphosate on zebrafish. Glyphosate was dissolved in the standard system water. To determine the diameter distribution and zeta-potential of the treatment concentrations in standard system water, the authors conducted the dynamic light scattering (DLS). Besides, the authors tested the pH of treatment concentrations with pen pH meter PHB-3(Shanghai) before glyphosate exposure.

Glyphosate exposure in zebrafish embryonic development
Glyphosate was purchased from Beijing Qinchengyixin Technology Co. Ltd (Beijing, China). Its purity was to 99.8%. Glyphosate was added to the standard system water to prepare stock solutions of 600 mg/L; and subsequently diluted to obtain different concentrations (600, 400, 200, 100, 10, 5, 1, 0.5, 0.1, 0.01 mg/L). The collected embryos were transferred to 6-well plate, 40 embryos in each well. The standard system water was removed from each well, and then appropriate dilution was added immediately. Glyphosate exposure was conducted at 0.75 hpf until 96 hpf and solutions were refreshed

daily. During this period, embryo mortality was recorded at 3, 6, 10, 24, 48, 72 and 96 hpf. The percentage of epiboly was counted at 10 hpf and embryo hatching rate was recorded at 48 and 72 hpf. All embryos were observed under a stereomicroscope (Olympus ZX-10, Japan) equipped with a digital camera (Canon) at 3, 6, 10, 24 hpf and photographed for morphological analyses. At 3, 10, 24 hpf, choosing three different concentrations (1, 10, 100 mg/L) to test the surface tension of embryo. At 10 and 13 hpf, four treatment groups (0.1, 1, 10, 100 mg/L) were fixed with 4% paraformaldehyde (PFA) for whole-mount in situ hybridization. At 48 hpf, six treatment groups (0.01, 0.1, 0.5, 1, 5, 10 mg/L) were rinsed and the cultured in clean standard system water until 96 hpf for diel activity behavior assay. All experiments were conducted three times and had a control group (0 mg/L).

Morphological analyses of the larvae
Glyphosate exposure was conducted until 96 hpf and solutions were refreshed daily. At 96 hpf, all survived larvae were anesthetized by immersed in tricaine for photography under a stereomicroscope. Larvae were immobilized in low density agar gels to ensure photographs were taken from a consistent orientation. The body length and the head and eye areas of the larvae were measured using ImageJ software.

The test of embryos' surface tension
Three different concentrations (1, 10, 100 mg/L) were chosen to test the ratio of tension at a normal and a treated embryos' chorion. And each concentration was divided into three groups whose treatment periods are 3, 10, 24 hpf, respectively. A normal embryo and a treated embryo were held by two capillaries of micromanipulator with similar and low pressure. The two embryos were moved to the position where they slightly contact and their symmetry axis were located on one line which was in the same horizontal plane and paralleled with micromanipulator movement trajectory. Then the right arm of micromanipulator moved 200 mm forward to induce shape change of chorion. The micrographs were took after 5 min of squeezing. The angles θ_l and θ_r between the tangent of chorion-chorion interface and the tangent of left or right chorion-water interface at the edge of interface were determined by using edge detection program.

Whole-mount in situ hybridization
Embryos of different groups (0, 0.1, 1, 10, 100 mg/L) were collected respectively at 10 hpf (for *ntl*) and 13 hpf (for *krox20* and *shh*) for whole mount in situ hybridization and fixed in 4% paraformaldehyde, pH 7.0, in phosphate-buffered saline solution overnight at 4 °C. 20 embryos were collected for test at every group. Each experiment was repeated three times. Plasmids containing *ntl*, *krox20* and *shh* were used for probes synthesis. Antisense probes and whole-mount in situ hybridization were performed.

Diel activity behavior assay of larvae
First it was decided to use six lower concentrations (0.01, 0.1, 0.5, 1, 5, 10 mg/L) to test the effects of glyphosate on zebrafish larval locomotor activity behavior. The authors established a video-tracking system to record their locomotor activity over 48 continuous hours. The larvae were pipetted into a 96-well plate, one larva per well. Each group tested twelve larvae. To minimise evaporation, the 96-well plate was sealed with a layer of plastic film and then put on an acrylic shelf. An infrared LED array was located below the shelf as a backlight source. An acrylic diffuser was placed above the infrared LED array. A camera (MV-VS078FM, MicroVision, Japan) with a fixed-angel megapixel lens (MP5018, Computer) was fixed on the top for vertical observations. A custom program was developed with Microsoft Visual Studio 2010 and OpenCV 2.4.3 to record the larval diel activity behavior. The algorithm was based on the subtraction of the adjacent frames. During the experiment, the whole system was in a room at a constant temperature of 28.5 °C and with illumination from 7:00 a.m. to 9:00 p.m.. The test began at 4dpf (days post fertilization) at 9:00 p.m..

Immunofluorescence
Whole-mount immunofluorescence was performed. Larvae (28 hpf) of different treated groups (0.01 - 10 mg/L) were collected and fixed overnight at 4 °C in 4% paraformaldehyde, pH 7.0, in phosphate-buffered saline solution, n = 7 larvae. After washing by PBST, the embryos were kept in cold acetone. After incubation in 10% sheep serum for 1 h, the larvae were immersed in a primary antibody Znp1 (1:400) antibody overnight at 4 °C. Following several washes with PBST, larvae were incubated with a

secondary antibody Cy3- labeled goat anti-mouse IgG diluted 1∶300. Finally Laser Confocal Scanning Microscopy was used to observe the expression of fluorescence.

Data analysis
One-way ANOVA was performed to assess differences between the treated groups and the control group. The authors used SPSS 21.0, Graphpad prism 6.01, MATLAB and Excel 2010 to obtain the statistical charts. The value for each parameter is the average of all of the repeated experiments. The data were presented as the mean ± standard error of the mean (SEM). $p < 0.05$ were considered to indicate a significant difference. And the statistical significances were set to $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Fast Fourier transform, edge detection and Watsone-William test are implemented on MATLAB.

Results

Glyphosate exposure caused developmental retardation and increased mortality rate in developing zebrafish
The dynamic light scattering (DLS) results showed that glyphosate didn't exist in the form of undissolved particles. The zeta potentials of glyphosate dissolved in standard system water were electronegative. The pH of the treatment solutions was about 7.0 when the concentration was <10 mg/L. But at higher concentrations (>100 mg/L), the solutions were acidic For example, the pH of solution was 3.9 ± 0.03 at 400 mg/L.
Glyphosate exposure was started at 0.25 hpf and end at 96 hpf. At the early 24 h, the development of embryos was recorded. No morphological alterations were found between the control group and the treated groups from 3 to 24 hpf at lower glyphosate concentrations (0.1, 1, 10 mg/L). From 6 to 10 hpf, obvious delay in the epiboly process by higher glyphosate concentration (>10 mg/L) was detected. And significant embryonic abnormality and mortality rates were detected.
Especially at 6 hpf, all embryos treated by 600 mg/L glyphosate were dead. Apparently, at 10 hpf, the epiboly process should be completed 100%. But the epiboly processes of some embryos at different concentrations were just up to 90%, even 80%. Delay in the epiboly process increased as glyphosate concentrations increased. The developmental retardation caused by glyphosate followed a concentration-dependent pattern at 10 hpf. With the development continuing, the morphological characteristics of the zebrafish embryos which were exposed with higher glyphosate concentrations (>10 mg/L) at 24 hpf showed developmental delay compared with the control group (0 mg/L). Embryonic death occurred mainly in the previous 24 h.

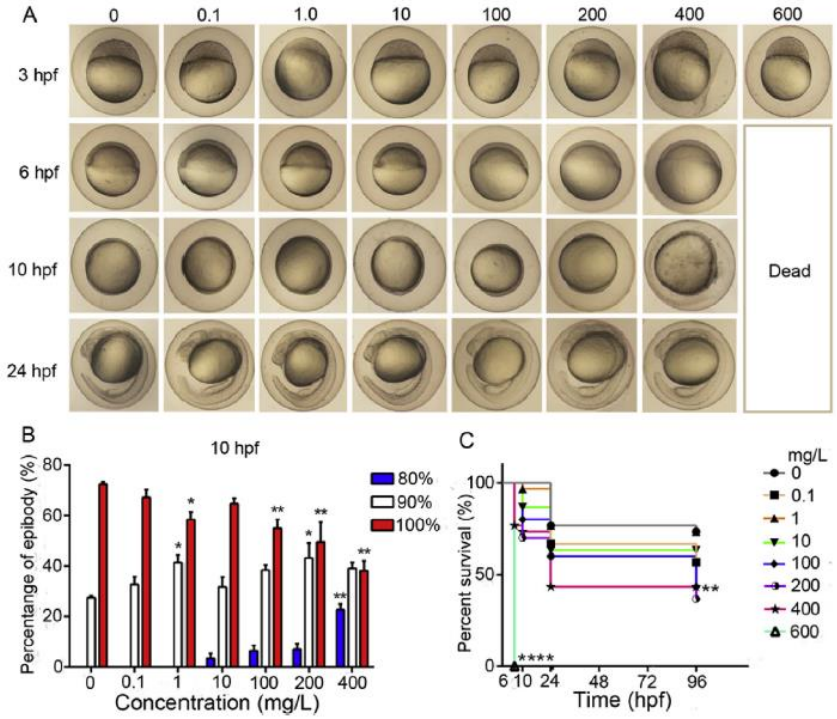


Fig. 1. Effects of glyphosate treatment on embryonic development. (A) Morphological characteristics of embryos exposed with glyphosate from 3 to 24 hpf. (B) The epiboly percentages of the embryos exposed to the glyphosate at 10 hpf. The error bar represents the standard error of the means (SEM). The statistical significances were versus the control group (C). The statistical significances were set to $p < 0.05$ (*) and $p < 0.01$ (**). (C) Survival evaluation: Kaplan–Meier plot representing the percent survival (%) that survived in 6 distinct time point: 6, 10, 24, 48, 72, 96 hpf. n = 3 replicate colonies per treatment and each replicate containing 40 embryos. *Indicate significant differences from control group (0 mg/L) survival. The statistical significances were set to $p < 0.05$ (*), $p < 0.01$ (**).

Surface tension of chorion decreased because of glyphosate treatment

According to toxicity of glyphosate in developing zebrafish, the authors chose a series of concentrations to test the ratio of tension at a normal (control group) and a treated embryo's chorion. When the test concentrations were less than 1 mg/L, the change of ration was not influenced by glyphosate (RMS notes that the data are not shown in this study). Therefore, the authors chose three (1, 10, 100 mg/L) treatment groups. The ratio of tension at two normal treated embryos' chorion approximately equal to 1 with approximately equal θ_l, θ_r. But the ratios of tension at a normal and a treated embryos' surfaces decreased with larger θ_l and larger difference between θ_l and θ_r as the concentration of glyphosate increased. At different treatment time, the ratio of tension at a normal embryo and a treated embryo was decreased significantly compared with two normal embryos, but there were no significant difference within different treatment concentrations. Furthermore, the decrease of the ratios became more significant with longer treatment periods, especially 24 hpf. In short treatment periods, the increase of θ_l and θ_l, keeping constant caused the ratios decrease. But in long treatment periods, the increase of θ_l, and decrease of θ_r caused the ratios decrease. It indicated that glyphosate caused decrease of the tension of embryo's chorion, but not at a concentration-dependent manner.

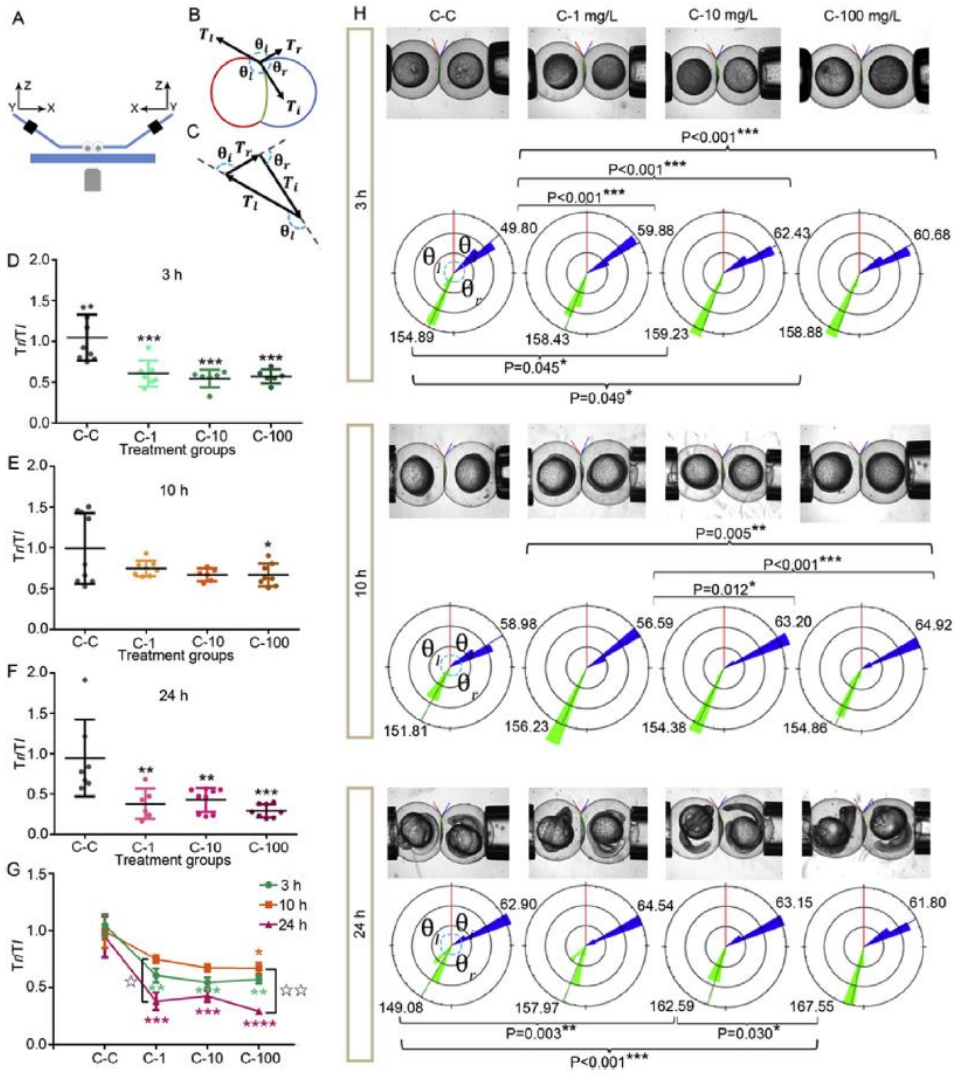


Fig. 2. Effects of glyphosate treatment on embryo surface tension. (A) Schematic diagram of experimental equipment. (B–C) Physical model of our test. There are three kinds of tensions exist on the edge of interface of two embryos, which are normal (left) chorion-water T_l, treated (right) chorion-water T_r, chorion-chorion T_i. The angles between the tangent of chorion-chorion interface and the tangent of left or right embryo-water interface at the edge of interface are θ_l and θ_r respectively. The angle between the tangents of two embryo-water surfaces at the edge of interface is θ_i. (D–F) The ratio of tension at two embryos' surfaces. "c" represents the control embroy. (G) The ratio of tension at two embryos surfaces at different time points. n ≈ 6. The error bar represents the standard error of the means (SEM). The statistical significances were set to $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $P < 0.0001$ (****). The symbol pentacle (☆) represents statistical difference profile between different treatment time. (H) Micrographs of tested embryos at their final position (The embryo at left was the control, which was not exposed glyphosate, while the embryo at right was exposed to different glyphosate concentration. The red line represents tension at left chorion-water interface T_l. The blue line represents tension at right chorion-water interface T_r. The green line represents tension at chorion-chorion interface T_i.) and distribution of tangents of three chorions' surfaces. The red line is the tangents of chorion's surface of normal (left) embryos and set to 0°. The blue sectors are the range of chorion's surface of treated (right) embryos' surface. The green sectors are the range of interface of two chorions. The dark blue line and dark green line are their mean orientations respectively. The number is angle of the mean orientation. The radius of sector is the proportion of surfaces lie in the range corresponding to the sector and the radius of the three circles are 0.25, 0.5, 0.75. The three angles between each of two tangents are θ_l, θ_r, θ_i. *Indicate significant differences between tangents. The statistical significances were set to $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Glyphosate induced hatching rate increase and larvae abnormality

To further explore the effect of glyphosate on developing zebrafish, morphological assessment and analysis were conducted for larval development at 96 hpf and hatching rate was recorded at 48 hpf and 72 hpf. From 100 to 400 mg/L, the glyphosate exposure resulted in shorter body lengths, smaller eyes and heads, especially in 400 mg/L, there were significant differences comparing to control group. Interestingly, exposure to higher concentrations of glyphosate increased hatching rate compared with the control group.

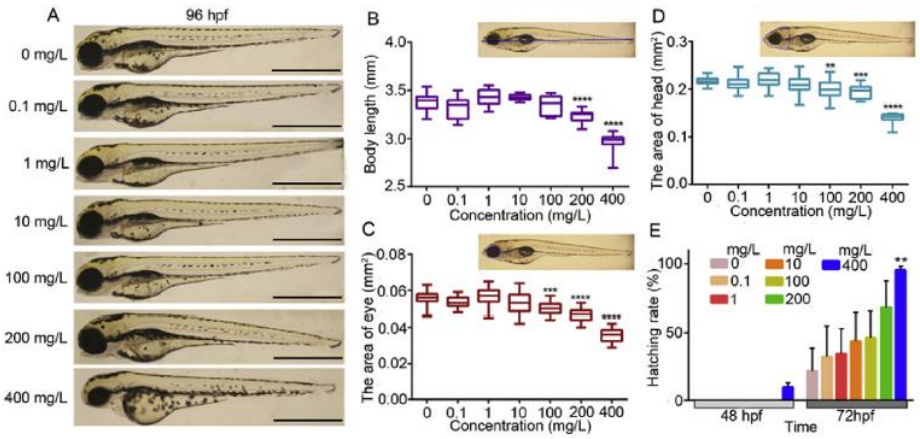


Fig. 3. Phenotypes of zebrafish larvae exposed to different concentrations of glyphosate and hatching rate of different time. (A) Morphological characteristics of control larvae and larvae exposed to different concentrations of glyphosate at 96 hpf. Scale bars = 1 mm. (B–D) Body length, eye area, head area of larvae. n = 88, 68, 88, 76, 52, 44, 52. (E) The hatching rate of larval treated with different concentrations at 48 and 72 hpf. n = 92, 80, 92, 76, 72, 72, 52. The error bar represents the standard error of the means (SEM). The statistical significances were set to $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

Glyphosate treatment changed gene expression patterns in embryo development

The morphological changes are closely related to gene expression patterns. The authors used Whole-mount in situ hybridization to test three key genes' expression, including *ntl*, *krox20* and *shh*. A part of concentrations (0.1, 1, 10, 100 mg/L) was chosen to assess the effect of glyphosate on zebrafish embryos. The *ntl* expression pattern in embryos treated with glyphosate was shorter than the control group as glyphosate concentrations increased. And the expression of *krox20* changed as glyphosate concentrations increased. Expression of *krox20* changed and the expression of in r5 was completely broken. However, the level of *shh* expression were spatially and temporally normal.

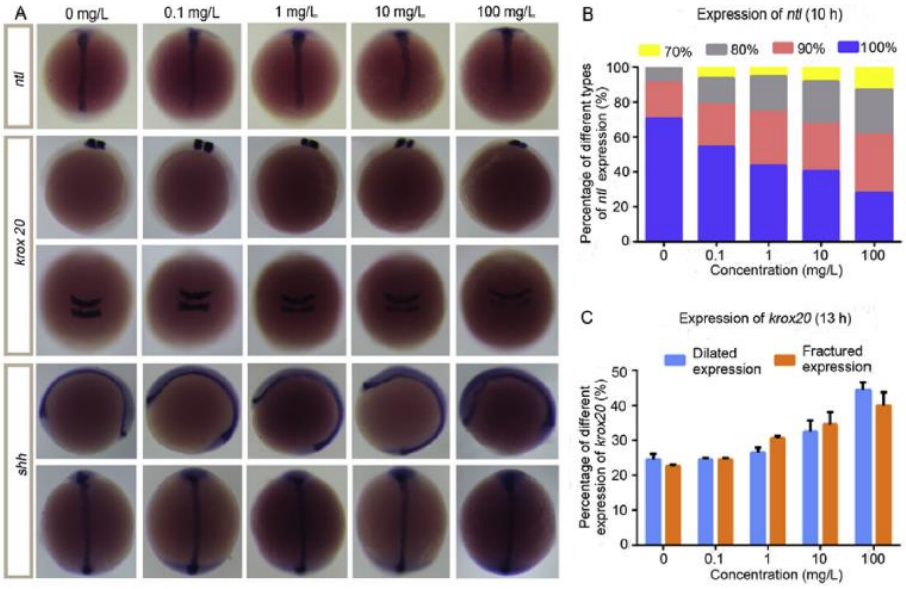


Fig. 4. Gene expression in embryos exposed to different concentrations of glyphosate. (A) Expression of *ntl* at 10 hpf, *krox20* at 13 hpf, and *shh* at 13 hpf in embryos exposed to 0 (C), 0.1, 1, 10, 100 mg/L glyphosate. (B) Statistical analyses of four types of *ntl* expression at 10 hpf in the controls and treated embryos. (C) Statistical analyses of two types of *krox20* expression at 13 hpf in the controls and treated embryos. The error bar represents the standard error of the means (SEM). n = 40 tested embryos.

Larvae treated with glyphosate increased their locomotor activities in day time

Considering the low toxicity of glyphosate to zebrafish in general case, the authors tested the diel activity behavior of zebrafish larvae which were exposure to lower glyphosate concentrations previously in their early embryonic development. Embryos were reared in various concentrations of glyphosate (0.01, 0.1, 0.5, 1, 5, 10 mg/L) to 48 hpf, then moved to control solutions until 96 hpf, then the test was run from 96 hpf to 144 hpf. The authors run the behavior test at 9:00 PM at 96 hpf. The authors utilized a high throughput, quantifiable approach to record the larval locomotor activity and could obtain the locomotor activity data of larval diel activity behavior for 48 continuous hours. From these data, they extracted five parameters, including the rest total, the rest bouts, the rest bouts length, total activity and waking

activity, to assess the diverse effects of different glyphosate concentrations. Based on the five normalized parameters, the authors conducted k-means clustering analyses according to shared behaviors. The authors chose k = 2 for the detailed analyses. The clustering analysis demonstrated that the lower and the higher concentrations induced different behavioral phenotypes. The lower concentrations increased activity and decreased rest to varying degrees on two days. But the higher concentration mainly affected the two parameters slightly on the second day. Based on the time series, glyphosate seemed no to significantly affect the two parameters on night time.

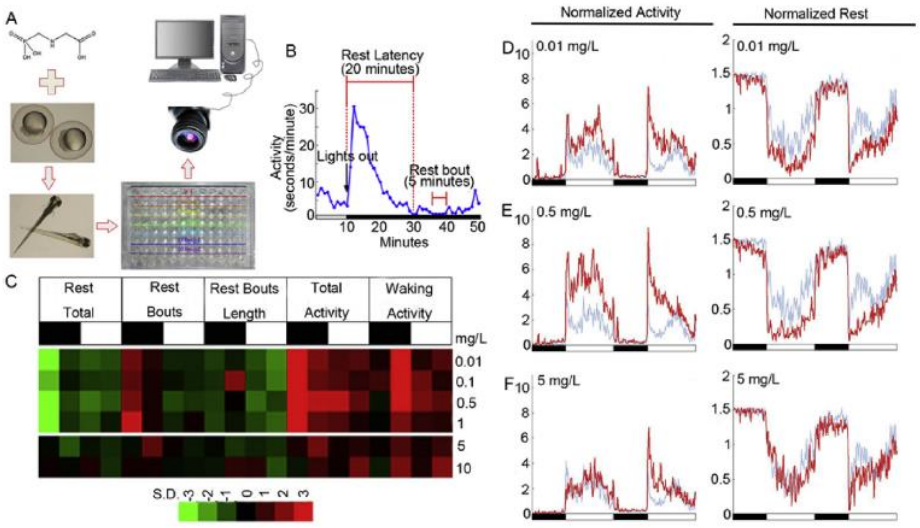


Fig. 5. Locomotor activity assay in larval zebrafish and behavioral similarities induced by different concentration of glyphosate. (A) The process for the diel activity behavioral assay. Glyphosate exposure was started at 0.25 hpf and ended at 48 hpf. Larva (4 dpf) was then pipetted into each well of a 96-well plate (~360 μL) with different concentrations of glyphosate at 9:00 a.m. Zebrafish were observed beginning at 9:00 p.m. at 4 dpf. (B) We defined five parameters, including rest total, total activity, the number of rest bouts, rest bout length and waking activity from locomotor activity of a representative larva. (C) K-means clustering analysis. Each row represents a concentration of glyphosate, and each column represents a behavioral parameter. The black bars indicate the night measurements, and the white bars indicate the day measurements. These parameters were normalized as standard deviations from the control values. The red and green colors indicate that the values are higher and lower than the controls, respectively. (D–F) The waking activity and the rest total were averaged in 10-min intervals and then normalized to the control values. In the time series analysis, the red trace indicates the average of the treated group, and the blue trace indicates the average of the controls. The black and white bars represent the night and day measurements, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In order to explore the more specific effects on larval behavior, five behavioral parameters were further quantitatively analyzed, including behavioral fingerprints, line graphs, histograms and Fast Fourier transform. Compared with control group, the exposure groups decreased rest total time on days and the first night significantly. Waking activity increased inordinately in all treated groups on 48 continuous hours. Glyphosate only increased number of rest bouts on the first night and decreased rest bouts length on the first night and day at 0.01-1 mg/L. Those two parameters changed irregularly and non-significantly. In short, glyphosate mainly increased larval activity on day time. The authors used Fast Fourier transform to investigate whether the zebrafish rhythms changed. All groups had the same main wave which had a 1200-min period and corresponded to the alternation of day and night. Compared with control group, the main wave of treated groups had greater amplitudes. Besides, the authors detected that many high-frequency but smaller-amplitudes waves which didn't exist in control group. It manifested that larvae exposed to glyphosate increased their locomotor activities, especially at 0.01-1 mg/L.

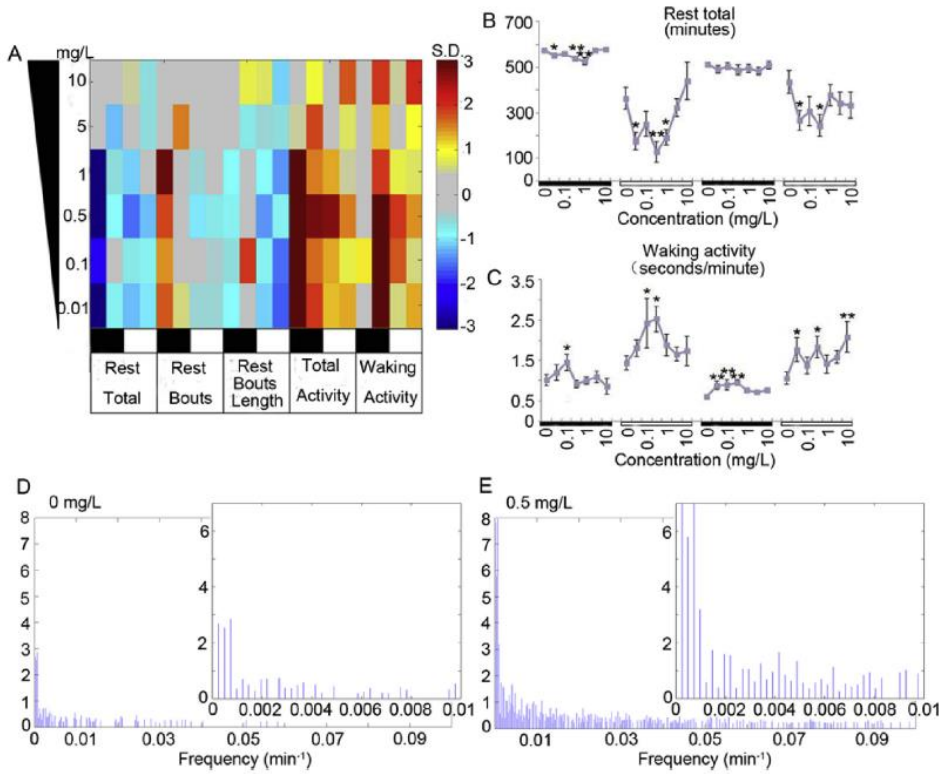


Fig. 6. Qualitative and quantitative analyses of behavioral changes induced by different concentrations of glyphosate. (A) In the fingerprint, five parameters were described. From left to right, the parameters were: rest total, number of rest bouts, rest bout length, total activity and waking activity. Each row represents a concentration of glyphosate, and each column represents a behavioral parameter. The measurements are normalized as standard deviations from the control values. The blue and red colors indicate that the values were lower and higher relative to the control values. The black bars indicate the night measurements, and the white bars indicate the day measurements. (B–C) In the line graphs for rest total and waking activity, each value indicates the average of ~36 larvae. The error bar represents the standard error of the means (SEM). The statistical significances were set to $p < 0.05$ (*) and $p < 0.01$ (**). The black and white bars indicate the night and day measurements, respectively. (D–E) Frequency domain graphs at concentrations of 0 and 0.5 mg/L. The graph in the top right corner is the detail of 0–0.01 min^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Glyphosate damaged motoneurons expression in larvae

Zebrafish have two types of spinal motoneurons, primary motoneurons (PMNs) and secondary motoneurons (SMNs). Primary motoneurons are born early in development, around the end of gastrulation, while secondary motoneurons arise later in development and are more numerous than PMNs. Here, the authors focused on one of the PMNs, CaP, which has a ventrally projecting axon. From control group, they could see the motoneurons expressed in a regular pattern. With the increase of glyphosate concentrations, motoneurons became less and shorter, especially in 10 mg/L.

Conclusion

The authors combined developmental assay with biomechanics and zebrafish behavior profiling to assess the biological effects of glyphosate on zebrafish. Development delay at concentrations higher than 100 mg/L from 3 hpf, and embryonic death mainly in the previous 24 h, showing glyphosate at higher concentrations could affect the early development of zebrafish in morphology. The high mortality of embryos was mainly attributed to the embryos' developmental retardation and abnormality. No tail (*ntl*) is one of important genes that are required for early notochord formation. In molecular level shown by *in situ* hybridization, the shorted expression of *ntl* demonstrated that glyphosate could reduce the structure of notochord, which is in accordance with the shorter body length in morphology. The expression of *ntl* in notochord in 10 mg/L and 100 mg/L groups were shorter than that of the wild typed embryos. But the expression of *shh* of 10 and 100 mg/L groups were same to those of the wild typed embryos at 13hpf. *Shh* was expressed at notochord at 13 hpf, which suggested that the development of notochord was not affected by glyphosate of 10 and 100 mg/L. Thus, the short expression of *ntl* only suggested that glyphosate of 10 and 100 mg/L could cause a delayed epiboly. The normal *shh* expression in notochord in embryos of 10 and 100 mg/L groups was in accordance with the body length in embryos of 10 mg/L and 100 mg/L, suggested that the glyphosate of 10 and 100 mg/L have no effect on the body length. The fractured expression of *krox20* demonstrated that glyphosate could change the gene

expression patterns and interrupted the hindbrain segmentation, especially in the formation and specication of rhombomeres 5, as glyphosate concentration increased. High glyphosate concentrations (>100 mg/L) could cause larvae malformation. Likewise, there were no obvious morphological changes at lower concentrations. At the mean time, the authors found that treatment solutions were acidic at concentrations higher than 100 mg/L. Glyphosate caused an obvious decrease in pH.

Based on the development tests, the authors chose three glyphosate concentrations to treat embryos, and test surface tension compared with a control group at 3, 10, 24 hpf. The results showed that surface tension of glyphosate treated groups decreased at a same time point in a non-concentration independent manner. As treated time extended, surface tension significantly declined, such as 24 hpf. However, no significant difference between 3 hpf and 10 hpf was found. In the authors' opinion, surface tension's reduction resulted from that glyphosate destroys the structure of chorion, leading to brittle and easily broken of chorion. The authors found that higher hatching rate was at 48 and 72 hpf than control embryos. It's speculated higher hatching in glyphosate treated embryos closely related with surface tension's reduction caused by glyphosate. Behavior test showed that the glyphosate of 0.01 and 0.5 mg/L could increase the locomotive activities in day time. The concentration of 0.01 mg/L is close to environmental concentration of glyphosate, indicating the environmental glyphosate could change organism behaviors and increase spontaneous movement. With the concentration of glyphosate increased, the primary motoneurons were damaged severely which may be correlated with the changed locomotive activities alterations caused by glyphosate.

Overall, high concentrations of glyphosate can induce zebrafish development delay and embryonic death. The surface tension of chorion after glyphosate treatment was decreased. The primary motoneurons, CaP axons, were damaged which may result in the increased locomotive activities alterations caused by glyphosate. The decreased surface tension of chorion and the increased locomotive activities may contribute to the hatching rates after glyphosate treatment.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Provides information on a test species that is relied upon in the EU ecotoxicological regulatory risk assessment, however the endpoints cannot be related to an EU level ecotoxicological regulatory risk assessment.

Assessment and conclusion by RMS:

Effects of glyphosate on early development of larval zebrafish were investigated via morphological, biomechanics, behavioral and physiological analyses.

The relevance of the parameters investigated in this study for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level (this latter being the specific protection goal). This study is nevertheless informative and is retained by RMS.

- NOEC for morphological alterations =10 mg/L (epiboly process and body length, eye and head area)
- NOEC Surface tension of chorion < 1mg/L (not concentration dependant), the study author claims that it is not significant at concentrations below 1mg/L but the data are not shown in this study
- NOEC hatching rate = 200mg/L (increase with concentration)
- NOEC larvae abnormality = 10 mg/L

Gene expression (these genes were not related to endocrine activity) and locomotor activity was not considered directly relatable to the risk assessment. A 48-h locomotion test revealed that embryonic exposure to glyphosate significantly elevated locomotor activities especially at 0.01-1 mg/L.
It is hypothetized that the decreased surface tension of chorion and the increased locomotive activities may contribute to the hatching rates after glyphosate treatment.

No standardized guideline was used. Dynamic light scattering (DLS) was used to confirm that no undissolved glyphosate powder was present in the test solutions, the accuracy and precision of the DLS method are unknown.
The study is relevant and reliable with restrictions.

B.9.2.2.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Lopes F. M. *et al.*
Report year	2014
Report title	Effect of glyphosate on the sperm quality of zebrafish *Danio rerio.*
Document No	Aquatic toxicology (2014), Vol. 155, pp. 322-6
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

In this study, the effect of glyphosate on sperm quality of the fish *Danio rerio* was investigated after 24 and 96 h of exposure at concentrations of 5 mg/L and 10 mg/L. The spermatic cell concentration, sperm motility and motility period were measured employing conventional microscopy. The mitochondrial functionality, membrane integrity and DNA integrity were measured by fluorescence microscopy using specific probes. No significant differences in sperm concentration were observed; however, sperm motility and the motility period were reduced after exposure to both glyphosate concentrations during both exposure periods. The mitochondrial functionality and membrane and DNA integrity were also reduced at the highest concentration during both exposure periods. The results showed that glyphosate can induce harmful effects on reproductive parameters in *D. rerio* and that this change would reduce the fertility rate of these animals.

Materials and methods

Adult zebrafish (*D. rerio*) males were obtained from commercial distributors and maintained in tanks containing dechlorinated and aerated water at 28 ± 2 °C, pH 7.0, 0 ppm of nitrite, under a photoperiod of 12 h light: 12 h dark. Fish were fed *ad libitum* with a commercial fish food twice a day. After three weeks of acclimation, the animals (total length: 37.9 ± 0.6 mm, weight: 0.541 ± 0.2) were randomly divided into three experimental groups (n = 27 per group). The experiments were performed in 2 L aquariums (three fishes per aquarium) with nine replicates. The water conditions during the experimental period were the same as during the maintenance period.

The control group was kept only in water and the exposed groups received glyphosate solutions at final concentrations of either 5 mg/L or 10 mg/L. Animals were exposed for 24 h or 96 h (water was renewed after 48 h, to keep water quality). It is important to note that no mortality was observed in the test groups during the exposure period. At the end of the exposure period, the animals were sacrificed, and the pair of testes of each fish were excised and placed in tubes containing 100 mL of Beltsville Thawing Solution (BTS) for subsequent analysis. The tubes were shaken for the release of spermatozeugmatas (sperm bundles). Sperm was released by gently and repeatedly disrupting the spermatozeugmatas with a 10 µL pipette tip. The sperm suspension was used for the analyses. In all subsequent applications, two hundred cells per animal were analyzed.

The sperm concentration, sperm motility and motility period were evaluated using a phase-contrast microscope with 200× magnification, after combining 1 µL of sperm diluted in BTS solution and 99 µL of control water (28 °C) in order to active the spermatozoa. The sperm concentration was measured using a Neubauer chamber, and the results were expressed as sperm per milliliter of semen. Sperm motility and the motility period were accessed on a slide covered with a coverslip. Sperm motility was expressed as the percentage of progressive motile spermatozoa 10 s after activation, and the motility period was comprised of the time (in *s*) between sperm activation and the absence of progressive movement (straight line movement). The mitochondrial functionality was evaluated according to He and-Woods adapted by Varela Junior *et al*., using a rhodamine 123 probe, which accumulates only in functional mitochondria. The mitochondria were considered functional when sperm presented positive rhodamine 123 staining (green fluorescence) and nonfunctional when sperm presented no fluorescence. The results are expressed as the percentage of sperm with functional mitochondria compared to total sperm.

The membrane integrity of the sperm was examined following the methodology of Harrison and Vickers. Fluorescence was verified at 400× magnification using an epifluorescence microscope. When the spermatozoa membrane was intact, CFDA accumulation occurred. After the hydrolysis of CFDA, carboxy-fluorescein was generated along with a corresponding green fluorescence. Sperm with damage in the membrane incorporated PI and emitted a red or red and green fluorescence. The percentage of sperm viability was determined by the proportion of sperm emitting green fluorescence compared with the total number of sperm (green, red or red and green).

Sperm DNA integrity was evaluated using the acridine orange method described by Varela Junior *et al*., where the meta chromatic colorant acridine orange emits green fluorescence in reaction to double-stranded DNA and an orange or red fluorescence in reaction to single-stranded DNA, identifying a break

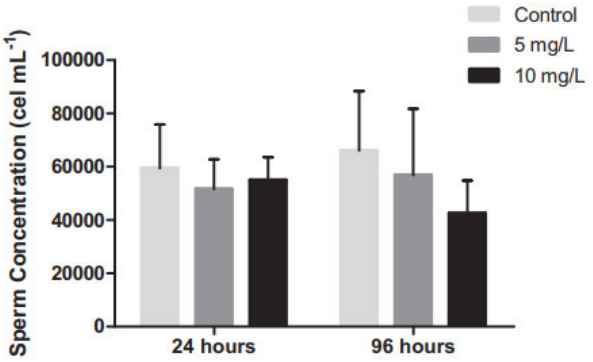
in the DNA.

Results were presented as the means ± standard error of the means (SEM). The statistical analyses were made using the parameters considered normally distributed and homoscedastic were tested by analysis of variance (ANOVA),comparing the three groups of animals exposed to glyphosate(0 mg/L, 5 mg/L and 10 mg/L), with comparisons of the means done by Tukey's test HSD with a significance level of 5%. The parameters without normal distribution were submitted to the Kruskal-Wallis analysis of variance for non-parametric data, followed by the Kruskal-Wallis all-pairwise comparisons, using a significance level of 5%.

Results

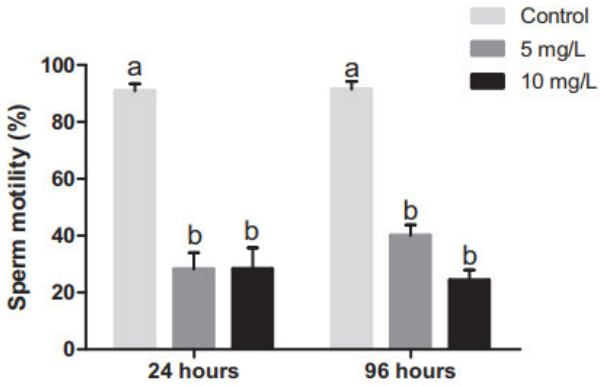
No significant differences were observed in sperm concentration between groups at any duration of exposure as indicated in Fig 1.

Figure 1. Sperm concentration in *Danio rerio* exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24–27). There was no significant difference among the treatments ($p < 0.05$).



Glyphosate exposure significantly reduced ($p < 0.05$) sperm motility and the motility period after both exposure periods and at both herbicide concentrations as indicated in Figure 2.
The control group had a sperm motility of 90.9% (±2.5) after 24 h and 91.4% (± 2.7%) after 96 h of exposure. Treatment with 5 mg/L of glyphosate resulted in a decrease of 62.8% and 51.4%, at 24 and 96 h, respectively. At a concentration of 10 mg/L, there was a decrease of 62.6% after 24 h and 67.1% after 96 h. No significant differences were observed in these parameters between glyphosate-exposed groups at the different exposure periods.

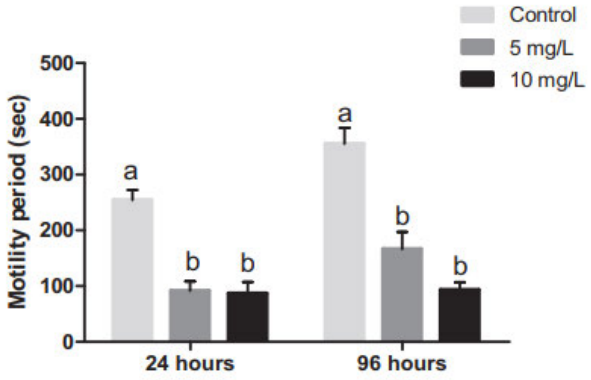
Figure 2. Sperm motility in *Danio rerio* exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24–27). The different letters represent significant differences among treatments at the same exposure periods ($p < 0.05$).



Concerning the sperm motility period, a 2.5-times reduction was observed with the 5 mg/L glyphosate

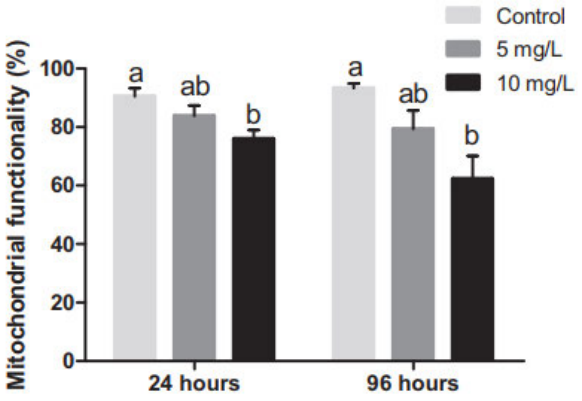
treatment, and a 2.9-times reduction was observed with the 10 mg/L glyphosate after 24 h. After 96 h of exposure, there was a decrease of 2 times in the 5 mg/L group and 4 times in the 10 mg/L group (Fig. 3). No significant differences were observed between groups exposed to glyphosate ($p > 0.05$).

Figure 3. The motility period, in s, in *Danio rerio* exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24-27). The different letters represent significant differences among treatments at the same exposure period ($p < 0.05$).



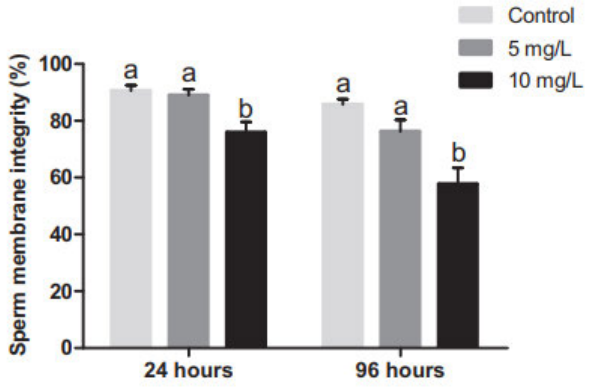
Mitochondrial functionality was significantly ($p < 0.05$) affected by glyphosate exposure ($p < 0.05$). Reductions of 20% (± 6.6%) and35% (± 7.7%) in mitochondrial functionality after 24 and 96 h of exposure, respectively, were observed in fishes exposed to 10 mg/L of glyphosate when compared with the control group (Fig. 4).

Figure 4. Sperm mitochondrial functionality in *Danio rerio* exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24-27). The different letters represent significant differences among treatments at the same exposure period ($p < 0.05$).



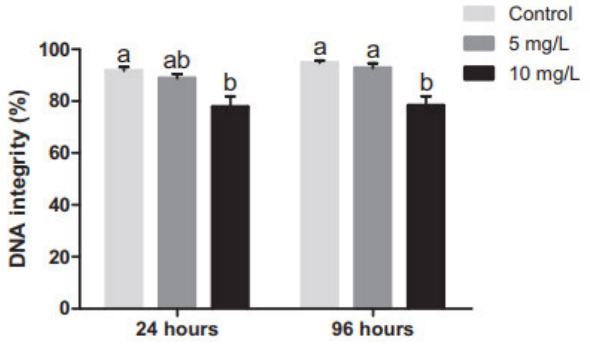
A significant decrease ($p < 0.05$) in the sperm membrane integrity of fish exposed to 10 mg/L of glyphosate was observed (Fig. 5). After 24 h of exposure, the membrane integrity was 75.8% (±3.7%) in this group, while in the control group and the group exposed to 5 mg/L, it was 90.5% (±1.8) and 88.9% (±2%), respectively. The same was observed after 96 h. The animals exposed to 10 mg/L of glyphosate presented a significant ($p < 0.05$) reduction in membrane integrity to 57.7% (±5.6) compared with the controlgroup (85.7 ± 1.9%) and the group exposed to 5 mg/L (76.2 ± 4%).

Figure 5. Sperm membrane integrity in Danio rerio exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24-27). The different letters represent significant differences among treatments at the same exposure periods ($p < 0.05$).



The DNA integrity was significantly compromised compared with the control group animals after 24 h of exposure to 10 mg/L of glyphosate (Fig. 6). A reduction of 14% was observed compared to the control group at this exposure period. After 96 h, the DNA integrity in the animals exposed to 10 mg/L of glyphosate was 78.3% (±3.5), which was significantly different from both the control group animals (94.7 ± 0.9%) and the group exposed to 5 mg/L of glyphosate (92.6 ± 1.9%).

Figure 6. DNA integrity in *Danio rerio* exposed for 24 h and 96 h to glyphosate (5 mg/L and 10 mg/L) and the respective control groups (0.0 mg/L). The values are means ± SEM (n = 24-27). The different letters represent significant differences among treatments at the same exposure periods ($p < 0.05$).



Conclusion

The results showed that glyphosate may induce harmful effects on the reproductive parameters of *D. rerio* males, such as damaging sperm DNA, reducing the integrity of the mitochondrial membrane and functionality, and decreasing sperm motility and the motility period. The alterations at the molecular level, such as reduction in DNA integrity and damage to membranes and mitochondrial functionality, are the causes of the cellular functionality impairment observed in terms of motility and the motility period. Taken together, these alterations would dramatically reduce the fertility rate of these animals, hindering reproductive success.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. geo-climatic properties, land-uses and agricultural practices differ from EU). The study presents no endpoints which can be directly used in the ecotoxicological regulatory risk assessment. The test substance is not sufficiently identified and exposure concentrations were not confirmed by chemical analysis.

Assessment and conclusion by RMS:

The effect of glyphosate on sperm quality of the fish Danio rerio was investigated after 24 and 96 h of exposure at concentrations of 5 mg/L and 10 mg/L. The spermatic cell concentration, sperm motility and motility period were measured employing conventional microscopy. The mitochondrial functionality, membrane integrity and DNA integrity were measured by fluorescence microscopy using specific probes.

- No significant differences in sperm concentration were observed;
- sperm motility and the motility period were reduced after exposure to both glyphosate concentrations during both exposure periods.
- The mitochondrial functionality and membrane and DNA integrity were also reduced at the highest concentration during both exposure periods.

The parameters investigated are considered relevant for the risk assessment. However the concentrations tested in laboratory conditions were high (5 mg/L and 10 mg/L) and above those expected in environmentally realistic conditions.
The test item is not clearly defined. No analytical verification available.
The study is considered less relevant but supplementary (due to the uncertainty on the test item) and reliable with restrictions.

Data point:	CA 9
Report author	Sulukan E. *et al.*
Report year	2017
Report title	An approach to clarify the effect mechanism of glyphosate on body malformations during embryonic development of zebrafish (*Daino rerio*).
Document No	Chemosphere (2017), Vol. 180, pp. 77-85
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

In this study, the effects of glyphosate were investigated on enzyme activity of carbonic anhydrase, production of reactive oxygen species, cell apoptosis and body morphology during the embryonic development of zebrafish. Embryos were exposed at 4 hpf to 1, 5, 10 and 100 mg/L glyphosate for 96 h. The survival rates, hatching rates, body malformations under the stereo microscope were evaluated in 24, 48, 72 and 96th hours. In order to clarify the mechanism of the abnormalities ROS, enzyme activity of carbonic anhydrase and cellular death were detected end of the 96th hour. The data obtained show that glyphosate treatment inhibited CA activity, caused production of ROS especially branchial regions, triggered cellular apoptosis and caused several types of malformations including pericardial

edema, yolk sac edema, spinal curvature and body malformation in a dose-dependent manner. As a conclusion, in light of present and previous studies, the researchers can deduce that (1) the probable reason of ROS production was CA inhibition via decreasing of CO_2 extraction and developing respiratory acidosis (however, one needs to clarify), (2) abundance of ROS triggered cellular apoptosis and (3) as a result of cellular apoptosis malformations increased.

Materials and methods

Zebrafish maintenance and embryo treatment

AB strain zebrafish (Danio rerio) were obtained from Oregon State University (US) and were kept in Aquatic Habitats (Imported by Akuamaks Co., Turkey) zebrafish system which was maintained a constant temperature of 28 °C under a 14:10 h light-dark photoperiod. The fish were fed with *Artemia salina* twice a day. Zebrafish embryos were obtained from spawning adults in groups of about 20 males and 10 females in tanks overnight. Spawning was induced in the morning when the light was turned on. Embryos were examined at 4 hpf (hours post fertilization) under a dissecting microscope, and unfertilized and death embryos were removed. Selected 40 embryos for each group, that had developed normally and reached the blastula stage, were treated graded concentrations of dosing solutions (1, 5, 10, and 100 mg/L) of glyphosate which were prepared in E3 embryo medium (5mM NaCI, 0.17mM KCI, 0.33mM CaCI2, 0.33 mM MgSO4, %0,01 methylene blue).

The commercial formulation of glyphosate (N-phosphono methylglycine, 360 mg/L) was used. Mediums renewed every 24 h. The concentrations were selected based on a previous studies (Topal et al., 2015), and information from the available literature (Webster et al., 2014; Lopes et al., 2014). Three replicates for each concentration were used. Mortality was identified by coagulation of the embryos, missing heartbeat, failure to develop somites and a non-detached tail (Shi et al., 2008). Dead embryos were recorded and promptly removed from the solution during observations. During the 96 h of exposure, embryos and larvae were examined under a stereomicroscope (Zeiss, Discovery V12, Germany) to screen for morphological abnormalities (included pericardial edema, yolk sac edema, body malformation and spinal curvature) and recorded at 24, 48, 72 and 96 hpf among the embryos and larvae from both the control and treated groups. 3% Methyl cellulose was used for immobilized larvae during imaging.

CA enzyme activity assay

For enzyme activity end of the treatment (at 96 h) randomly selected 10 larvae from each group (control, 1, 5, 10, and 100 mg/L) were homogenized via Tissue Laser (Qiagene) in appropriate amount Tris-HCl buffer, then centrifuged at 10,000g for 30 min at 4 °C, supernatant was used for activity determination. CA enzyme activity was assayed by following the hydration of CO2 according to the method described by Wilbur and Anderson (1948). CO_{2-} hydratase activity as an enzyme unit (EU) was calculated by using the equation (t0-tc/tc) where t0 and tc are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively (Alım et al., 2015; Huyut et al., 2016).

Reactive oxygen species (ROS) detection

Reactive oxygen species was detected in 10 larvae selected randomly from each group (control, 1, 5, 10, and 100 mg/L) at 96 hpf. The compound chloromethyl-20,70-dichloro dihydrofluorescein diacetate (CM-H_2DCFDA, Calbiochem) was used to monitor the accumulation of reactive oxygen species in live

zebrafish larvae. This molecule reacts with many different ROS, and it is a general indicator of oxidative stress (Invitrogen). Fluorescent DCF was formed through ROS oxidation. Selected larvae were washed with ultra-pure H_2O three times, and excess water was removed (Kim et al., 2014). Larvae were incubated in 1 µg/mL concentration and 2mL amount of CM-H_2DCFDA for 2 h under dark condition and washed again with ultra-pure H_2O three times, then immobilized in 3% methylcellulose (Kim et al., 2014). Images of live samples were captured using a fluoresce stereo microscope (Zeiss, Discovery V12, Germany).

Apoptosis detection

Apoptotic cells were detected in 10 larvae selected randomly from each group (control, 1, 5, 10, and 100 mg/L) at 96 hpf using acridine orange (AO) staining, a nucleic acid selective metachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attractions (Hu et al., 2011). Larvae were rinsed three times with PBS and incubated in 5 mg/mL concentration and 2 mL amount of AO for 30 min in the dark at 28 °C, followed by three times rinses in PBS (Duan et al., 2013). Stained larvae were examined using fluorescence microscopy (Zeiss, Discovery V12, Germany). Whole larvae fluorescence was measured and quantified using Volocity Demo 6.3 software (PerkinElmer, USA) (Duan et al., 2013).

Results

Survival and hatching rate of embryo/larvae

The survival and hatching rate were determined to evaluate possible toxicity of glyphosate on zebrafish developmental stage. As shown in Fig. 1, survival rate decreased dose dependent, but approximately over %60 of embryo can success live in all treatments groups. No statistical differences were observed between treatment groups but all treatment groups have significantly decreasing ($p < 0.05$) compared to that of control group in survival rate. There was no significantly differences between all groups except highest dose in hatching rate (Fig. 2). According to these results, the study authors state that glyphosate has delaying effect on hatching rate in 100 mg/L concentration in zebrafish embryo.

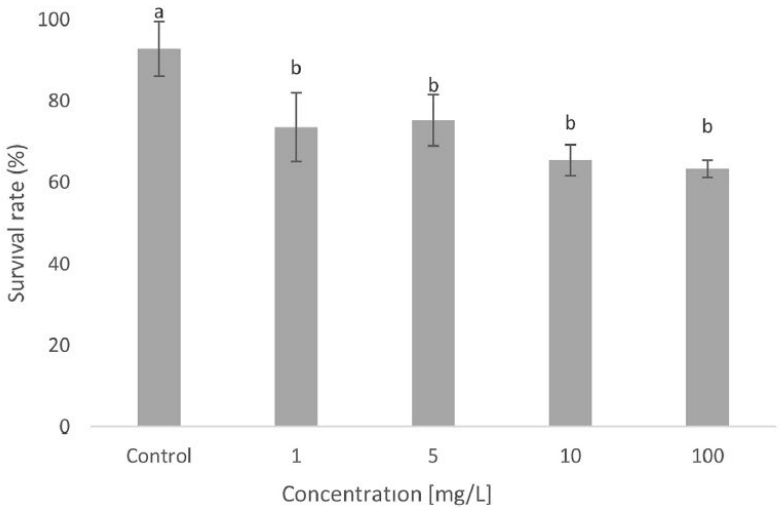


Fig. 1. Survival rate of zebrafish embryos induced by glyphosate. Data are expressed as means ± S.D. from three independent experiments. Different letters indicate significant differences between the groups ($p < 0.05$; ANOVA, Tukey's test). ($n = 3$; 40 embryos for each n).

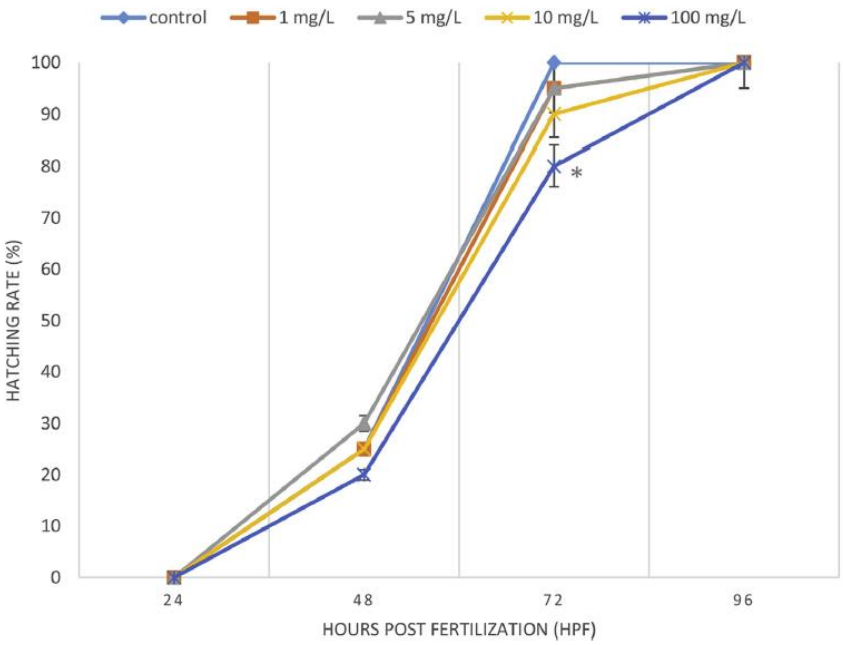


Fig. 2. Hatching rate of zebrafish embryos induced by glyphosate for 4–96 hpf. Data are expressed as means ± S.D. from three independent experiments (*$p < 0.05$).

Malformations of embryo/larvae

The malformations triggered by glyphosate in zebrafish embryo and larvae was recorded (Figs. 3 and 4).

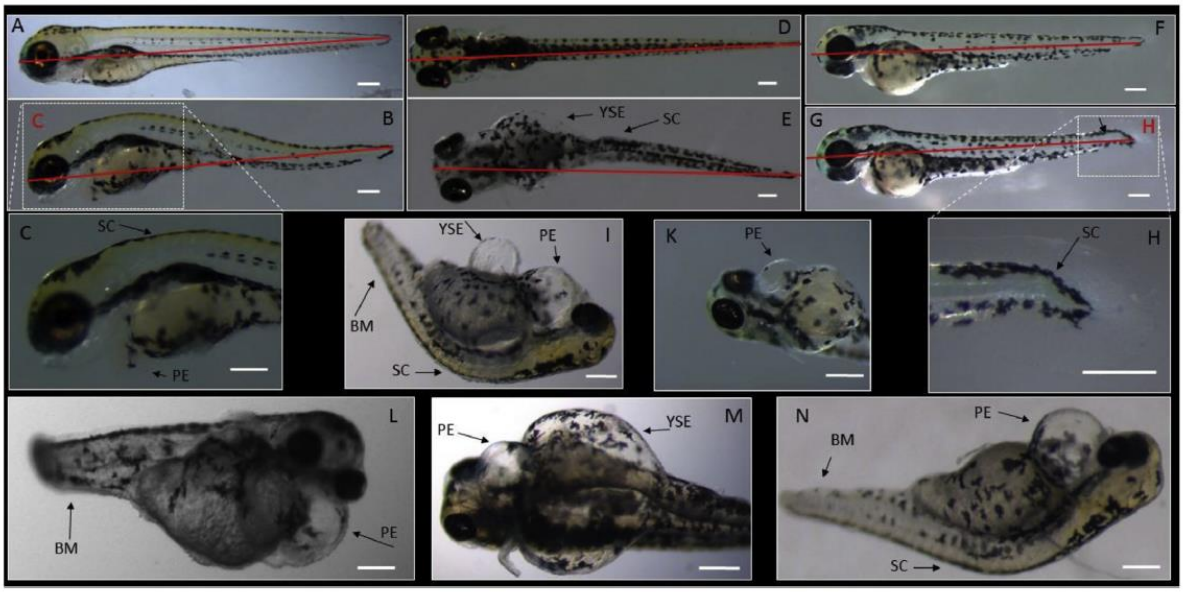


Fig. 3. Microscopic images of embryos. Control embryos (A (96 hpf), D (96 hpf) and F (72 hpf)); 10 mg/L glyphosate treatment groups (B and C (72 hpf), E (96 hpf), G and H (72 hpf)); 100 mg/L glyphosate treatment groups (I and L (72 hpf), K, M and N (96 hpf)). YSE: yolk sac edema; PE: pericardial edema; SC: spinal curvature; BM: body malformations (including tail malformation, short tail and head malformation). Scale bar: 200 μm.

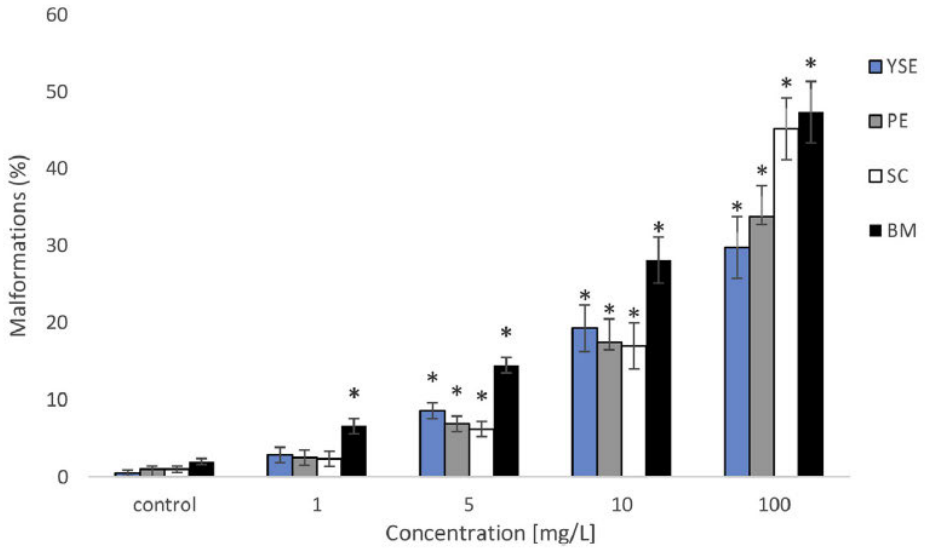


Fig. 4. Malformation of zebrafish embryos exposed to glyphosate. Data are expressed as means ± S.D. from three independent experiment (*p < 0.05).

The typically malformations including pericardial edema (PE), yolk sac edema (YSE), body malformations (BM) (including tail malformation, short tail and head malformation) and spinal curvature (SC) were searched in all treated and control groups at 24, 48, 72 and 96 hpf during the 96 h exposure time. Body malformation had highest percentage among the recorded malformations in all treatment groups. There was significantly difference even in lowest dose (1 mg/L) in body malformation ($p < 0.05$), although no significant difference was found from those in other malformations in 1 mg/L concentration. There was not found significant difference between control and 1 mg/L concentration groups in terms of all malformations except body malformation, but all malformations were more serious in embryo and larvae exposed to 100 mg/L concentration (Figs. 3 and 4).

CA enzyme activity

The CA enzyme activity was measured at the end of exposure time (96 h) from five larvae selected randomly each groups. As shown in Fig. 5, the results indicated that the activity was significantly inhibited in a dose-dependent manner in all treatment groups against control ($p < 0.05$). Although the strongest inhibition was found in 100 mg/L concentration group, no significant difference was found between 10 and 100 mg/L concentration groups.

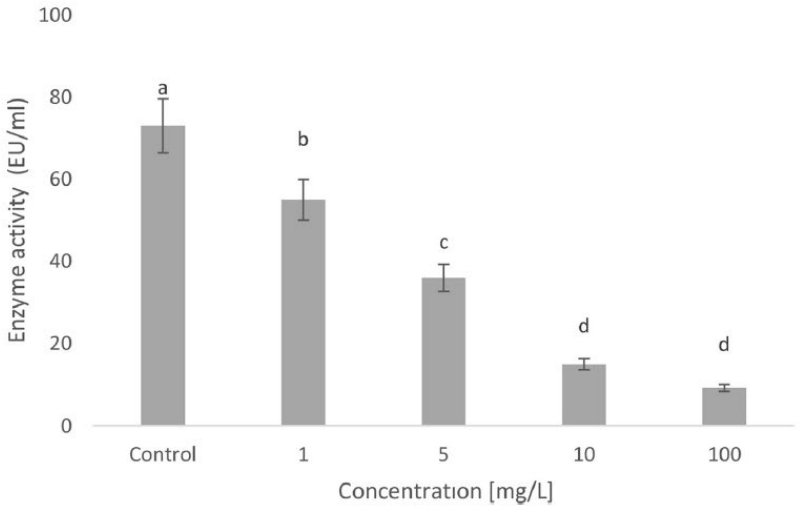


Fig. 5. Carbonic anhydrase enzyme inhibition by glyphosate. Data are expressed as means + S.D. from three independent experiment. Different letters indicate significant differences between the groups ($p < 0.05$; ANOVA, Tukey's test). ($n = 3$; 10 larvae for each n).

ROS detection

The researchers used a fluorescent ROS indicator, CM-H_2DCFDA, to determine whether the glyphosate produce ROS in vivo. This indicator produces a fluorescent green product when oxidized by ROS. They detected some fluorescent signal from different parts of the body such as eye, head, muscle, gut and intestine especially highest treatment dose, but in this study they focused signals comes from brachial regions (Fig. 6) to associate them with their CA enzyme inhibition results.

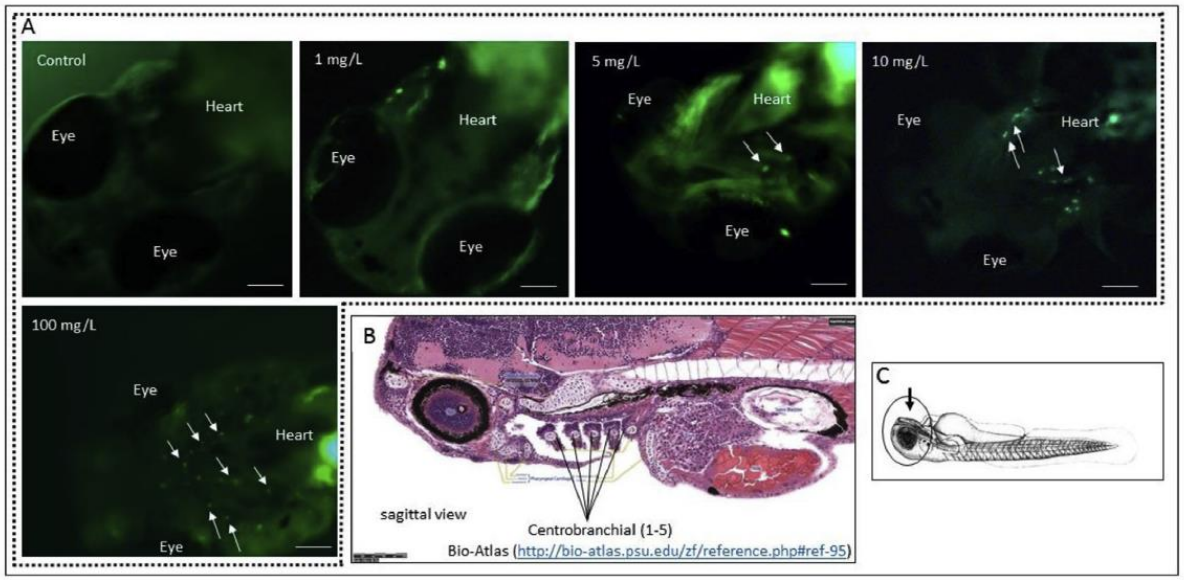


Fig. 6. CMH_2DCFDA staining in vivo production of ROS in gills (A). Scale bar: 600 μm. Panel B shows the location of gills in larvae 96 hpf. This figure (B) taken from Bio-atlas website; http://bio-atlas.psu.edu/zf/reference.php#ref-95. Panel C shows the position and location of larvae when imaging.

Apoptosis detection

Acridine orange (AO) apoptotic cells determination assays were performed to determine whether exposure to glyphosate would lead to an increase in cellular death in whole live larvae (Fig. 7A).

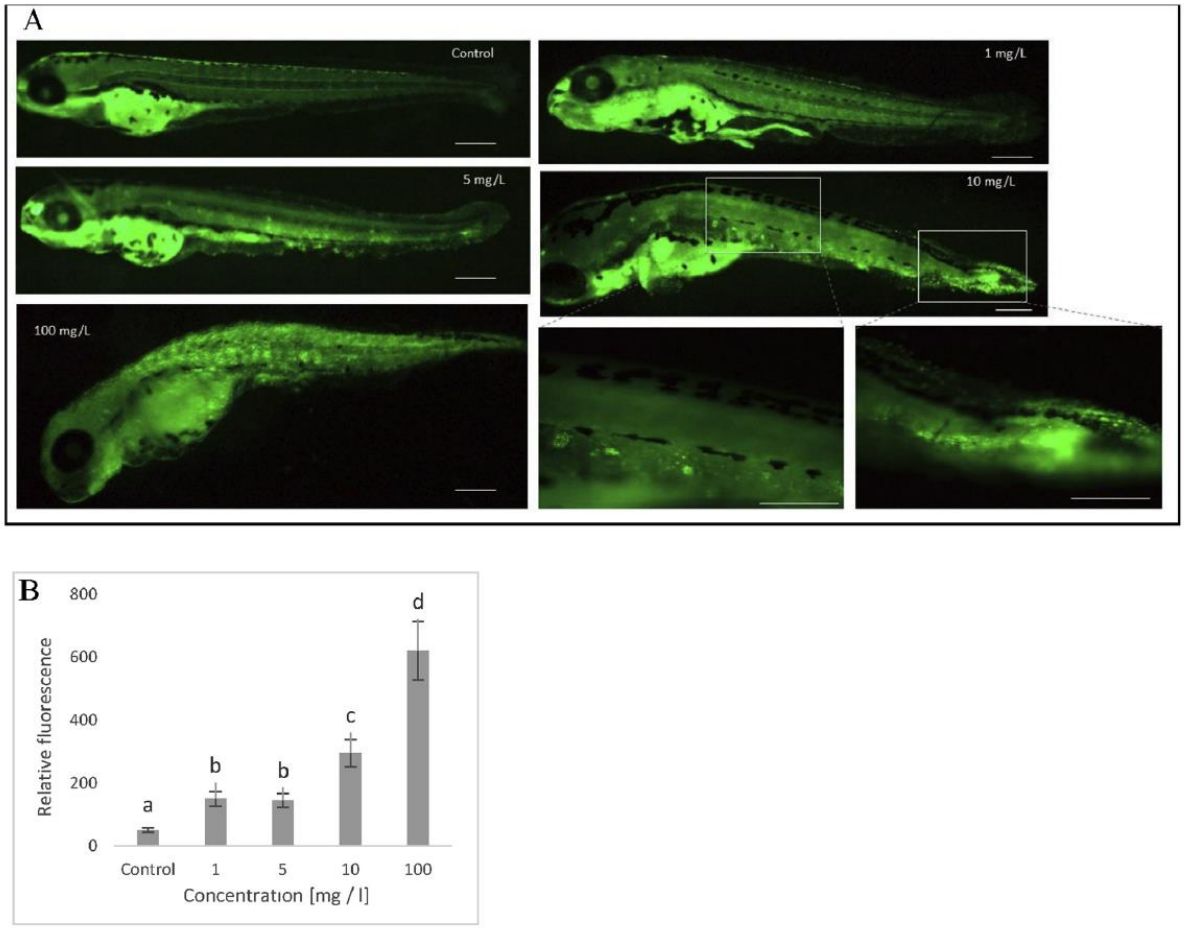


Fig. 7. (A) Apoptotic cells were determined using acridine orange staining of glyphosate treated larvae at 96 hpf. Whole larvae cell death images detected by fluorescence microscope. Scale bar: 200 μm. (B) The relative fluorescence of death cell. Data are expressed as means ± S.D. from three independent experiments. Different letters indicate significant differences between the groups ($p < 0.05$; ANOVA, Tukey's test). ($n = 3$; 10 larvae for each n).

The researchers observed that glyphosate treatment was resulted significantly increase in overall cellular death a dose-dependent manner (Fig. 7B), but a dramatically increasing was in highest concentration (100 mg/L) (Fig. 7A and B). The whole-larvae fluorescence analysis was confirmed with the incidence of their other results (malformations, CA enzyme activity and ROS detection) (Figs. 4-6).

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotox risk assessment.
In addition, it is not possible to confirm the identity of the formulation used in the study as only the following is mentioned in the paper: "*The commercial formulation of glyphosate (N-phosphono methyl-glycine, 360 mg/L) was used*". The surfactant system in the formulated product used in this study was not stated. It is therefore not possible to confirm whether the product used, is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

In this study, the effects of glyphosate in a formulation (not identified) were investigated on enzyme activity of carbonic anhydrase, production of reactive oxygen species, cell apoptosis and body morphology during the embryonic development of zebrafish. Embryos were exposed. The survival rates, hatching rates, body malformations under the stereo microscope were evaluated.
The main objective was to explain the underlying mechanism of the abnormalities. ROS, enzyme activity of carbonic anhydrase and cellular death were detected end of the 96th hour. The data obtained show that glyphosate treatment inhibited CA activity, caused production of ROS especially branchial regions, triggered cellular apoptosis and caused several types of malformations including pericardial edema, yolk sac edema, spinal curvature and body malformation in a dose-dependent manner.
The study authors associate the observed body malformations with cellular apoptosis caused by ROS and inhibition of CA, as a result of glyphosate treatment.
These effects were observed even at lowest concentration tested of 1mg/L.
However the formulation was not identified. Only the following is mentioned in the paper: "The commercial formulation of glyphosate (N-phosphono methyl-glycine, 360 mg/L) was used". In view of the formulation description, the use of the results to assess the toxicity of glyphosate as formulated in MON52276 is questionable.
RMS notes that no analytical verification was conducted. RMS also notes that effects on embryos survival was not concentration dependant and were at comparable (high) levels among all tested concentrations (except control). Hatching success seems also high, 100% success at 100 mg/L. RMS doubts the reliability of the results on these parameters. The study nevertheless showed significant effects on malformations (concentration dependant including lowest concentration of 1 mg/L), indicating that zebrafish embryos are sensitive to glyphosate exposure.

RMS considers that this study is less relevant but supplementary (due to formulation issue) and reliable with restrictions for use in risk assessment purpose.

B.9.2.3. Potential for endocrine disruption

B.9.2.3.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.2.1
Report author	Le Mer C. et al.
Report year	2012
Report title	Effects of chronic exposures to the herbicides atrazine and glyphosate to larvae of the threespine stickleback (*Gasterosteus aculeatus*).
Document Source	Ecotoxicology and environmental safety (2013), Vol. 89, pp. 174
Guidelines followed in study	none
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

as provided in the AIR5 dossier (KCA 9) as proposed by applicant: **See RMS analysis in RMS comment box**	

Full summary of the study according to OECD format

Threespine Stickleback (*Gasterosteus aculeatus*) adults from a clean reference site reproduce in the laboratory and the fertilized eggs were incubated until hatching. Larval sticklebacks (< 24 h old) were exposed for 42 days to four concentrations (0.1, 1, 10 and 100 µg/L) of glyphosate. A seawater control and positive controls for estrogenic (0.05 µg/L ethinylestradiol, EE2) and androgenic (3 µg/L dihydrotestosterone, DHT) effects. The survivors were measured (length, wet weight) then conserved for biochemical (VTG, vitellogenin a female egg yolk protein precursor, and the male nest-protein spiggin, SPG) and histological (phenotypic sex determination) analyses.
After 42 days exposure of larval sticklebacks to glyphosate concentrations (0.1, 1, 10 and 100 µg/L), there were no significant effects on larval survival or growth while exposure to 3 µg DHT/L resulted in a significant effect on growth (body lengths) but did not induce SPG. VTG was induced after the EE2 exposure, but glyphosate did not induce production of VTG and SPG. Exposure of early life stages of stickleback to sublethal concentrations of glyphosate did not cause a change in sex ratio or in the prevalence of mixed sex individuals. The proportion of mixed sex individuals was higher in the positive controls compared to the negative controls.

Materials and methods

Stock population
In May 2007, adult three-spined sticklebacks, *G. aculeatus*, were collected from an uncontaminated site on the Gaspe Peninsula (Riviere Saint-jean, Quebec, Canada) by minnow cages. Mature adult males and females displaying secondary sexual characters were separated and placed in 105 L tanks supplied with a constant flow of filtered seawater. Animals were acclimated over a 10-day period to a temperature of 18 ± 1 °C and salinity 15 ± 1‰, with a 16:8 light/dark photoperiod. Fish were fed twice a day with live or frozen adult Artemia (Artemia sp) and with a mixture of commercial fish pellets (Nutrafin®, Rolf C. Hagen Inc, Montreal, QC, Canada) at approximately two percent body weight per day.

Reproduction
After 10 days of acclimation, 40 males and females were placed in 45 L glass tanks containing sand and filamentous algae as nest-building material. Mating trials were conducted with several males and a minimum of five females. After mating, fertilized egg clutches were removed from the nest and transferred to 1 L beakers, with aerated IML seawater water (18-19 °C) until hatching.

Chemicals
Glyphosate (purity ≥ 96%), DHT (purity ≥ 99%) and EE2 (purity ≥ 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Chronic laboratory exposures
Experiments were conducted during summer 2007 and repeated during summer 2008.
Exposures began at hatching of larvae (7 days after fertilization). Newly hatched larvae in good condition were selected and exposed to different treatments in 1 L glass beakers in 500 mL seawater supplied with aeration at 18 °C with a photoperiod of 16:8 light/dark. Larvae were exposed to four nominal concentrations (0.1, 1.0, 10 and 100 µg/L) of glyphosate, to a seawater control and to positive controls, EE2 (0.05 µg/L) and DHT (3 µg/L). Each treatment included 3 replicates with 9-10 fish each, a total of 27-30 animals per treatment.
Larvae were fed once a day with *Artemia* nauplii. Dissolved oxygen, temperature and fish mortality, determined by lack of reaction to gentle prodding, were monitored daily in all exposures. Water was changed every day (> 80% replacement rate) before feeding. Animals were exposed throughout their

embryo-larvae and juvenile life stages (from hatching to 42 dph) until the juveniles had a mean length of approximately 20 mm.
At the end of the 42-d exposure period, surviving larvae were sacrificed and lengths (to the nearest 0.1 mm) were measured under a dissecting microscope. Half of the individuals from each replicate, selected randomly, were conserved for measurements of VTG and SPC (i.e., 4-6 larvae from each replicate, 13-18 total). The remaining individuals of each replicate were preserved for the evaluation of intersex (13-18 total).

Preparation of exposure solutions
Glyphosate was dissolved directly in deionised water. The exposure solutions were prepared in 20 L bottles by appropriate dilutions of stock solution and stored in the dark at 4 °C. EE2 and DHT solutions were prepared every day.
Glyphosate samples were analyzed in the 2008 experiment only (n=3). In 2007 glyphosate concentrations in exposure and stock solutions were intended to be measured by ELISA (Abraxis LLC, Warminster, PA, USA). However, the analyses of spiked solutions and the samples showed high variability and low sensitivity.
In 2008, analyses of glyphosate was conducted by a provincial government laboratory, the Centre d'Expertise En Analyse Environnementale Du Québec (2008). Samples were collected once a week and placed in 500 mL plastic bottles supplied by the CEAEQ, with 100 mg/L of $Na_2S_2O_3$ added to neutralize chlorine. Samples were kept at 4 °C and shipped on ice to the CEAEQ laboratory in Quebec City within 48 h of collection. Glyphosate was analyzed by liquid chromatography, post-column derivation and fluorescence detection, with a detection limit of 0.04 µg/L.
Samples for analyses of EE2 and DHT were collected over the course of the experiment in freshly prepared exposure solutions and in the same solutions after 24 h (n=8). 3 mL volumes from each exposure replicate were pooled and filtered (Acrodisc 1 µm glass fibre membrane filters, Canada wide Scientific Ltd, Ottawa, ON Canada). The filtrate was collected and frozen at -80 °C until analyzed with specific immunoassays (EE2: Japan EnviroChemicals Ltd, Tokyo, Japan; DHT: dbc Inc., London, ON, Canada). Detection limits were 0.05 µg EE2/L and 6.0 pg DHT/L.

Growth measurements and reproductive endpoints
On day 42, all stickleback larvae were sampled and anesthetised by immersion in MS-222 (0.1 g/L, ethyl 3-aminobenzoate methane sulfonate salt). Total body lengths (to nearest 0.1 mm) and wet weights (to nearest 0.01 mg) were measured and condition factor was calculated as: $CF = (W/L^3)100$ where W is the weight (in mg) and L is the length (in mm) of larvae. Half of the individuals, selected randomly from each treatment, were frozen in liquid nitrogen and stored at -80 °C for analyses of VTG and SPG.

Purification and characterization of vitellogenin (VTG)
Stickleback VTG (sVTG) from plasma of mature female fish was precipitated with EDTA and MgCl2 and isolated by anion exchange and gel filtration chromatography. All purification steps were performed at 4°C with buffers containing the protease-inhibitor aprotinin (0.04TIU/ml) to reduce degradation of VTG. Protein contents were determined by the Bradford reaction (Bio-Rad Protein Assay, Bio-Rad Laboratories, Mississauga, ON, Canada).

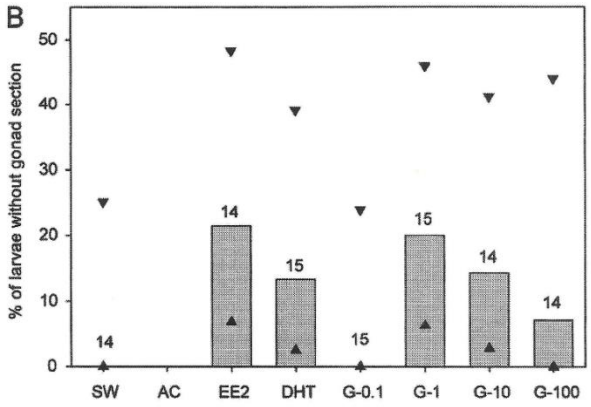
Stickleback-VTG competitive ELISA
VTG of stickleback was measured by an indirect competitive ELISA. For whole-body homogenates, the ELISA has a working range of 30-2000 ng VTG/mL and a limit of detection of approximately 15 ng VTG/mL.

Stickleback-spiggin (SPG) competitive ELISA
The competitive ELISA technique was performed using an antibody against a synthetic peptide specific to a spiggin sequence and spiggin standard made with hypertrophied kidneys from male sticklebacks exposed to androgenic substances. Levels of spiggin samples were measured colorimetrically with the substrate TMB at 450 nm using a microtiter plate reader. The detection limit was 0.5 U/mL.

Histological evaluation
At the end of the exposure, a subsample of randomly selected larvae (N=4 - 6 per replicate for a total of

12 -18) were fixed embedded in paraffin. The gonads were examined for phenotypic sex determination by light microscopy. The number of gonad sections per individual and the proportion of individuals without gonad sections did not differ among treatments (Fig. 1).
A gonad was determined as an ovary when the entire gonad was packed with oocytes. A gonad was determined as testis when it did not contain oocytes but spermatogonia-like germ cells packed in lobules. Control fish were used as reference for the normal appearance of juvenile testis and ovary. The presence of a sperm duct was not used as a character for identification of testis since it could rarely be observed on gonad sections. The presence of an ovarian cavity was not used as a character for identification of an ovary since it was not visible on small sections of female gonads. Gonads with an appearance intermediate between ovary and testis, containing at least one oocyte, but showing also a testis-like structure or spermatogonia-like germ cells in clusters, were determined as ovotestis.

Figure 1. Proportions of stickleback without histological sections of gonads for the glyphosate experiment (B). Triangles are 95% confidence intervals. Sample sizes are indicated at the top of the bars. There was no significant difference among treatments within experiments (Fisher's exact test, $p < 0.05$).



Statistical analyses
All variables were tested for normality (Shapiro-Wilk's W test) and homogeneity of variances (Levene test, Brown-Forsythe test) and square root, logarithmic, or arcsine transformations were applied when necessary. Differences among treatments were tested using analysis of variance (two-way ANOVA, with replicates nested in treatment blocks) or Kruskall-Wallis tests (Statistica Version 7.0, Statsoft Inc.) with an α=0.05 level of significance. In 2007, responses in seawater control and EE2 solutions were compared by *t*-test.
For histological analyses, the percentages of females or of mixed sex individuals were compared among treatments using the Fisher's exact test for significance ($p < 0.05$).

Results

Treatment concentrations
In 2008, mean glyphosate concentrations (± S.E.M.) were 0.08, 1.0 (± 0.6), 8 (± 1) and 78 (± 7) µg/L, reasonably close to the nominal values of 0.1, 1.0, 10 and 100 µg/L. The mean concentration (± S.E.M.) of EE2 was 0.06 ± 0.01 in both 2007 and 2008. Measured EE2 in fresh and 24-h exposures were > 80% of the nominal concentration. In contrast, concentrations of DHT in fresh solutions were only approximately 50% of nominal (3.0 vs 1.7 µg/L) and after 24 h were < 10% of the nominal value (mean of 0.20 µg/L). Concentrations of pesticides in the control seawater and acetone solutions were below detection limits.

Survival and growth endpoints
There was little mortality (< 11%) in the glyphosate exposures over the 42-day experiments.
In 2007, there were no significant differences in growth of juveniles (wet weights, lengths) after 42 days in the separate controls ($p < 0.05$) so the data for each set was pooled. Within each year, the body masses, lengths and condition of juveniles were similar in the glyphosate exposures compared to the appropriate controls (acetone carrier and seawater).

There was a significant difference in the size of individuals used in 2007 compared with 2008, despite the fact that the fish came from the same field site. At the end of the experiment in 2008, the juveniles were smaller (up to twelve percent less in body length) and weighed less (up to 32% in wet weight) compared to the animals in the 2007 experiment (Tables 1 and 2).
In the experiments of 2007 and 2008, there were significant differences in condition factor between EE2 exposed larvae and larvae in other experimental treatments (Tables 1 and 2). In 2007, the EE2 exposed group larvae differed with two glyphosate exposures (0.1 and 1 μg/l), the DHT controls and the seawater control. In 2008, the EE2 exposed group differed from two glyphosate treatments (1 and 100 μg) and the DHT controls.

Table 1: Mean (±S.E.M.) body lengths (mm), wet weights (mg) and condition factors (CF of larvae: (weight [mg]/length [mm]^3)*100) of fish on day 42 of the 2007 experiments. There were no significant differences for lengths, weights and condition factor between treatments (all same letter A). Glyphosate (GLY), positive controls 17α-ethinylestradiol (EE2) or dihydrotestosterone (DHT) and negative controls seawater (SW).

Treatment	Wet weight	Length	Condition factor
SW	76.8 ± 3.8	20.8 ± 0.4	0.83 ± 0.01 A
EE2	77.6 ± 2.3	21.4 ± 0.2	0.78 ± 0.01 A
DHT	74.4 ± 1.7	20.9 ± 0.2	0.82 ± 0.01 A
GLY 0.1 μg/l	75.0 ± 2.6	20.9 ± 0.2	0.81 ± 0.01 A
GLY 1.0 μg/l	75.6 ± 2.0	21.0 ± 0.2	0.81 ± 0.01 A
GLY 10 μg/l	76.6 ± 2.8	21.1 ± 0.3	0.81 ± 0.01 A
GLY 100 μg/l	77.0 ± 2.0	21.3 ± 0.2	0.80 ± 0.01 A

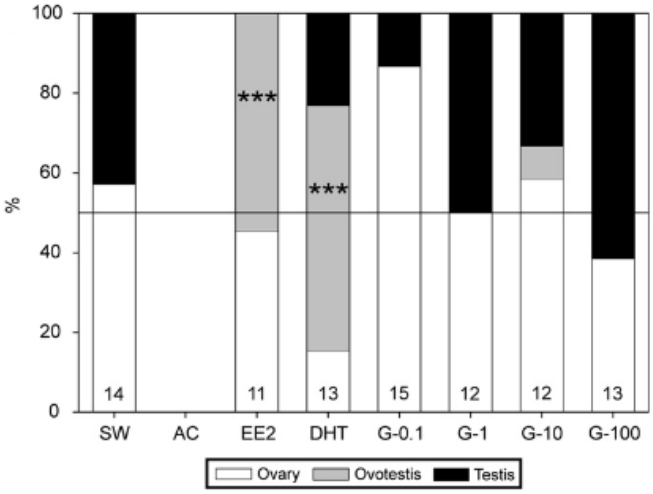
Table 2: Mean (±S.E.M.) body lengths (mm), wet weights (mg) and condition factors (CF of larvae: (weight [mg]/length [mm]^3)*100) of fish on day 42 of the 2008 experiments. There were no significant differences for lengths and weights between treatments. Different letters indicate significant differences between treatments for condition factor (Kruskall-Wallis test, $P < 0.05$). Glyphosate (GLY), positive controls 17α-ethinylestradiol (EE2) or dihydrotestosterone (DHT) and negative controls seawater (SW).

Treatment	Wet weight	Length	Condition factor
SW	52.4 ± 2.2	18.7 ± 0.3	0.79 ± 0.01 A B
EE2	49.0 ± 1.8	18.5 ± 0.2	0.76 ± 0.01 A
DHT	55.9 ± 2.5	18.8 ± 0.3	0.82 ± 0.01 B
GLY 0.1 μg/l	51.0 ± 2.6	18.4 ± 0.3	0.81 ± 0.01 A B
GLY 1.0 μg/l	53.6 ± 2.7	18.6 ± 0.4	0.81 ± 0.01 B
GLY 10 μg/l	51.2 ± 2.1	18.6 ± 0.3	0.79 ± 0.01 A B
GLY 100 μg/l	48.7 ± 1.7	18.2 ± 0.2	0.82 ± 0.04 B

Induction of VTG and SPG
There was a significant induction of vitellogenin (VTG) in juveniles exposed to 0.05 μg EE2/L compared to the other treatments ($p < 0.05$), with whole body VTG ranging from 30 ng/mg to 3.5 μg/mg body weight. No induction of VTG was observed in larvae exposed to glyphosate. Levels of VTG in larvae exposed to DHT and the negative control were below the detection limit of the assay (15 ng VTG/mL). No induction of spiggin (SPG) was observed in any treatment, including the positive DHT control.

Histological evaluation
Glyphosate had no significant effect on the proportion of mixed sex individuals. One mixed sex individual was found in the group exposed to 10 μg glyphosate/L and none in the seawater control. The proportion of mixed sex individuals was higher in the positive controls compared to the negative control. The proportion of female individuals did not differ among treatments. Proportions of females tended to be higher in fish exposed to the lowest dose of glyphosate and lower in fish exposed to DHT (Fig. 2).

Figure 2. Sex ratios in stickleback exposed to 0.1, 1, 10 and 100 µg/L of glyphosate, positive controls (l7α-ethinylestradiol (EE2) or dihydrotestosterone (DHT)) and negative control (seawater (SW). ***differ from SW control. Proportions of ovotestis and of females were compared among treatments within experiments with a Fisher's exact test, $p < 0.05$. Sample sizes are indicated at the bottom of the bars.



Glyphosate exposure caused no significant effects on survival, wet weight, length or condition of the larvae, at concentrations ranging from 0.1-100 µg/L.
In 2007 and 2008, a small non-significant effect of DHT exposure on larval growth (body length, wet weight) compared to controls was observed. Differences in wet weights ranging between 22 and 37%.
With the exception of DHT, measured concentrations were maintained at approximately 80% of nominal concentrations. Measured concentrations of DHT in freshly prepared solutions were approximately 50% of the nominal value of 3.0 µg/L, while those in the aged (24h) exposure solutions were below 20% of nominal (an average of 0.2 µg DHT/L). Levels of spiggin in the juvenile sticklebacks exposed to glyphosate for 42 d were below the detection limit of 0.5 U/mL, even in the DHT positive control.
While the exposure concentration of DHT did not induce elevated levels of spiggin; intersex in the positive DHT controls was observed.
The 42 d exposure to 0.05 µg EE2/L had a definite estrogenic effect on juvenile sticklebacks, with elevated levels of whole-body VTG (ranging from 30 to 3500 ng/mg).
Glyphosate induced no production of VTG in larval sticklebacks after 42-d exposures.
Exposure of early life stages of stickleback to sublethal concentrations of glyphosate did not cause a change in sex ratio or in the prevalence of mixed sex individuals. One individual with ovotestis was observed in the glyphosate treatments and none in the controls.

Conclusion

After 42 days exposure of larval sticklebacks to glyphosate concentrations (0.1, 1, 10 and 100 µg/L), there were no significant effects on larval survival or growth while exposure to 3 µg DHT/L resulted in a significant effect on growth (body lengths) but did not induce SPG possibly because of DHT degradation after the 24h solution renewal. VTG was induced after the EE2 exposure, but glyphosate did not induce production of VTG and SPG. Exposure of early life stages of stickleback to sublethal concentrations of glyphosate did not cause a change in sex ratio or in the prevalence of mixed sex individuals. The proportion of mixed sex individuals was higher in the positive controls compared to the negative controls.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The glyphosate analytical concentrations were highly variable, but overall based on the 2008 dataset, the mean measured values were within 25% of the nominal exposure concentrations. The sticklebacks were obtained from the natural environment and therefore prior exposure to chemicals cannot be discounted, although the fish were selected from the same location in two different years and achieved similar assay results in both years. The test system was considered robust based on the performance of the two positive control groups. Concerning the test design, the study was conducted according to methods described in Hahlbeck (2004) 'The juvenile threespined stickleback (*Gasterosteus aculeatus L.*) as a model organism for endocrine disruption: I. Sexual differentiation' whilst all available information is presented in this paper, the environmental conditions employed during the chronic exposure part of the test are not confirmed and validity criteria are not clearly stated. The achieved measured concentrations were also lower than is required for this study type and analysis in one of the two studies described was not complete. Given some of the uncertainty over elements of the test design, the study is considered unreliable.

Assessment and conclusion by RMS:

The aim of this study was to determine the effect of exposures to the common herbicides atrazine and glyphosate to larval sticklebacks (Gasterosteus aculeatus), using growth, survival and the reproductive biomarkers VTG, SPG and sexual differentiation as endpoints in larvae exposed to herbicides.

Less than 24h larval were exposed for 42 days to four concentrations (0.1, 1, 10 and 100 µg/L) of glyphosate.

Exposures began at hatching of larvae (7 days after fertilization) to 42 days after until the juveniles had a mean length of approx. 20 mm. Seawater was used. RMS notes that this species in also found in Europe in freshwaters.

Wild-caught specimens were used. These were collected from an "uncontaminated" site on the Gaspe´ Peninsula (Riviere Saint-Jean, Quebec, Canada) by minnow cages. RMS notes that it cannot be verified how far from the sea these fishes were caught (estuary or in the river, effect of salt?) but RMS assumes that contamination by pesticides should be rather low as the river seems mainly surrounded by non agricultural areas (mainly forest, based on satellite view on the web). RMS considers the uncertainty related to prior exposure to chemicals negligible. RMS however highlights that seawater was used in the test, interactions between the ions and the active substance cannot be discounted.

In 2008, mean glyphosate concentrations (± S.E.M.) were 0.08, 1.0 (± 0.6), 8 (± 1) and 78 (± 7) µg/L, therefore within 25% of the nominal exposure concentrations. Data for 2007 are lacking.

The study is well described and considered robust by RMS but did not follow standardized guideline, no validity criteria exist.

This study concludes that glyphosate does not show estrogenic or androgenic effects to early life stages of sticklebacks at environmentally realistic concentrations (0.1, 1, 10 and 100 µg/L). No induced production of vitellogenin or a change in sex ratio in early life stages of sticklebacks was observed.

This study is relevant for the assessment of the potential for endocrine disruption.

RMS considers this study relevant and reliable with restrictions.

B.9.2.3.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Armiliato N. *et al.*
Report year	2014
Report title	Changes in ultrastructure and expression of steroidogenic factor-1 in ovaries of zebrafish *Danio rerio* exposed to glyphosate.
Document No	Journal of toxicology and environmental health. Part A (2014), Vol. 77, No. 7, pp. 405-14
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

The aim of this study was to evaluate the toxicity of glyphosate on ovaries of zebrafish (*Danio rerio*). Ovaries (n = 18 per triplicate) were exposed to 65 µg/L of glyphosate for 15 d. This concentration was determined according to Resolution 357/2005/CONAMA/Brazil, which establishes the permissible concentration of glyphosate in Brazilian inland waters. Non-exposed ovaries (n = 18 per triplicate) were used as control. Subsequently, morphology and expression of steroidogenic factor-1 (SF-1) of exposed and non-exposed ovaries was determined. No apparent changes were noted in general morphology of exposed and non-exposed ovaries. However, a significant increase in diameter of oocytes was observed after exposure to glyphosate. When ovarian ultrastructure was examined the presence of concentric membranes, appearing as myelin-like structures, associated with the external membranes of mitochondria and with yolk granules was found. After glyphosate exposure, immunohistochemistry and immunoblotting revealed greater expression of SF-1 in the oocytes, which suggests a relationship between oocyte growth and SF-1 expression. These subtle adverse effects of glyphosate on oocytes raised a potential concern for fish reproduction.

Materials and methods

Test organism treatment and study design
Experiments were carried out using adult females of *D. rerio*. Specimens were exposed for 15 d to a concentration of 65 µg/L of glyphosate (Monsanto do Brasil Ltda) diluted in the aquarium water (n = 18 per triplicate). Non-exposed females were used as controls (n = 18 per triplicate). During exposure aquaria were checked daily to monitor water quality and survival. No mortality was observed throughout the 15 d exposure period. Following treatment, ovaries were extracted and weighed, and gonadosomatic index (GSI) was obtained. For morphometric analysis, diameters of germ cells (oogonia, previtellogenic, and vitellogenic oocytes) were measured and number of germ cells per area (NA) was determined.
Dewaxed ovary sections were incubated with primary polyclonal immumoglobulin G (IgG) rabbit SF-1 antibody. The sections were subsequently incubated for with anti-rabbit IgG peroxidase conjugated and binding sites of antibodies were revealed with 3,3'-diaminobenzidine and attributed to transmission electron microscopy.
Extracts from ovaries were used for SF-1 expression with polyclonal IgG rabbit SF-1 antibody. For detection, immune-complexed membranes were probed with anti-rabbit IgG peroxidase conjugated

antibody. The results of blots were expressed as ratio between intensities of SF-1 and β-actin bands. Membranes were probed with polyclonal IgG mouse β-actin antibody. For detection, immune-complexed membranes were probed with anti-mouse antibody and the intensity of the bands was quantified.

Data analysis

The quantitative results in terms of germ cells diameter and number of germ cells per area (NA) were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's HSD test using Statistica 6.0. Statistical analysis for SF-1 expression was per- formed using the computer package SPSS for statistical descriptive and Student's t-test.

Results

Morphology and Morphometry of Ovaries

Oogonia, previtellogenic I and II oocytes and vitellogenic oocytes were observed in exposed and non-exposed fish. No structural morphological differences in germ cells were observed between ovaries of exposed and non-exposed control females. The measurements of female germ cells showed that the 65 μg/L glyphosate used in this study did not markedly affect the diameter of the oogonia and the previtellogenic II oocytes. However, this same concentration induced a significant increase in the diameter of previtellogenic I oocytes compared to ovaries of non-exposed females. A marked rise in germ-cell diameter induced by glyphosate was observed in the vitellogenic oocytes compared to non-exposed females. The elevation in germ-cell diameter was also accompanied by higher GSI observed in the glyphosate-exposed females indicating that these ovaries reached a more advanced maturation stage. With respect to stereological parameters, no marked differences in number of germ cells per area (NA) between ovaries of females exposed to glyphosate and ovaries of non-exposed females were noted.

Ultrastructural parameters

Females exposed to 65 μg/L glyphosate showed ultrastructural alterations. The main changes were the presence of myelin-like structures, which are associated with mitochondrial external membranes and with yolk granules in the cortical regions of the oocytes concentric membranes. No apparent alterations were observed in other cytoplasmic organelles or in the nucleus of oocytes of exposed females. Similarly, no differences were detected in follicular cells or in vitelline envelope surrounding the oocytes.

Expression of SF-1

Females exposed to glyphosate showed greater expression of SF-1 as evidenced by immunohistochemistry labelling and immunoblotting. Non-exposed females displayed a lower reactivity to the antibody anti-SF-1 compared to females exposed to 65 μg/L glyphosate. Although females used for immunoblotting analysis showed similar means of GSI, the densitometric analysis indicated a higher expression of SF-1 in ovaries of exposed females. The SF-1 is involved in the establishment of the hypothalamic-pituitary-adrenal-gonadal axis and plays an essential role as a regulator of gonadal functions in vertebrate species. However, when targeted by an endocrine disrupting substance, one should expect a decrease of SF-1 expression, not an increase as observed in the current study.

Conclusion

Based on changes observed in SF-1 expression and on subcellular ultrastructure the authors concluded that glyphosate potentially affects reproduction of fish.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological risk assessment.
In addition, the Monsanto Brasil glyphosate formulated product used in the study was not sufficiently described. It is therefore not possible to confirm whether the product used, is the representative formulation MON 52276 relevant for the glyphosate EU renewal. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The aim of this study was to evaluate the toxicity of glyphosate on ovaries of zebrafish (Danio rerio). Effects of glyphosate (test item not clearly identified) on morphological and expression of steroidogenic factor-1 (SF-1) in fish ovaries were investigated. Fish were exposed for 15 days at one concentration: 65 µg glyphosate/L
No apparent change in general morphology, significant increase in diameter of oocytes was observed, immunohistochemistry and immunoblotting revealed greater expression of SF-1 in oocytes. Glyphosate induced subcellular and molecular impairments that may affect reprodution in females fish.
No NOEC was derived (only one concentration was tested)
Co-formulants are not stated. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. No detailed results are reported (only graphics).
Overall, the study is considered less relevant but supplementary (due to uncertainty on the test item) and reliable with restrictions.

B.9.2.4. Acute toxicity to aquatic invertebrates

B.9.2.4.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

No study belong to this category. However, datagap has been identified.

B.9.2.4.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Mugni H. *et al.*
Report year	2014

Report title	Acute toxicity of roundup to the nontarget organism *Hyalella curvispina*. Laboratory and field study
Document No	Toxicological and environmental chemistry (2014), Vol. 96, No. 7, pp. 1054-1063
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

Glyphosate is the most used pesticide in Argentina. *Hyalella curvispina* is a widely distributed and commonly abundant component of the invertebrate assemblages in shallow waters of southern South America. The aim of this study was to assess the acute toxicity of the increasingly common Roundup Full II®, commercial formulation of the herbicide glyphosate (66.2% active ingredient), to *H. curvispina* in laboratory and field assessments. The mean estimated 48-h LC_{50} of Roundup Full II® was 9.9 ± 1.7 mg/L. In a field experiment Roundup Full II® was applied to soybean plots. Simulated rain was generated the following day by means of irrigation sprinkler equipment. *H. curvispina* was exposed to runoff water and soy leaves. No mortality was observed. It is suggested that Roundup Full II® crop applications represent a low risk of acute toxicity to *H. curvispina* adults inhabiting water bodies adjacent to crop fields.

Materials and methods

LC_{50} determination
The 48-h LC_{50} of glyphosate to *H. curvispina* was determined on six independent occasions during a nine-month period between May 2011 and February 2012. Specimens of *H. curvispina* were collected from an uncontaminated stream located 25 km south of La Plata city and transported to the laboratory, where they were reared for several weeks. They were kept in large plastic containers with stream water, which was gradually replaced with un-chlorinated tap water to compensate for evaporation losses. The locally abundant macrophyte Lemna sp. was placed on the surface of the water. *H. curvispina* specimens fed on the periphytic community of the *Lemna rhizosphere* and received a supplement of a mixture of fresh lettuce leaves and separate cultured algae twice a week.
Procedures for *H. curvispina* toxicity tests were adapted from standardized protocols for soil toxicity tests for *H. azteca* (US EPA 2000), as described by Mugni et al. (2013). Ten *H. curvispina* specimens, 5-10 mm in length, were exposed to different glyphosate concentrations in 100 mL of reconstituted, moderately hard synthetic water (APHA 1998), placed in 250-mL beakers. Three replicates of each concentration were tested. Tests were performed without feeding, at 22 ± 2 °C, and natural photoperiod. Dead individuals were removed immediately. Mortality was recorded at 48 h of exposure. As a validity criterion for the negative control, less than 10% was considered acceptable. Preliminary tests were conducted to choose an appropriate glyphosate concentration range within which to test lethal effects. As a standard laboratory quality control practice, a reference test with copper sulfate ($CuSO_4$ * 5 H_2O, 99.9%, Merck, Darmstadt, Germany) was performed. The 48-h LC_{50} positive control was 265 µg/L Cu(II). This value lies within the acceptable range in the control chart (225 ± 79 µg/L Cu(II)) conducted by Mugni (2009). Toxicity tests were performed using Roundup Full II®, formulation (66.2% active ingredient). A stock solution of Roundup (133 mg/L) was prepared with reconstituted moderately hard

water (APHA 1998). Different exposure solutions were prepared by diluting the stock solution in reconstituted moderately hard water. Three replicates were performed. Nominal assayed glyphosate concentrations were 25, 20, 15, 10, 7, 5 and 2 mg/L. In the first LC_{50} determination glyphosate concentrations in replicates of the 2, 5 and 10 nominal doses were determined after a 2-h exposure; measured concentrations were 1.6, 3.6 and 8.7 mg/L, respectively. The LC_{50} concentrations were calculated taking into account recovery-corrected concentrations. Organisms were considered dead when no response was observed upon gentle prodding. Mortality data obtained from the 48-h exposures were used to estimate the LC_{50} and its 95% confidence limits by means of the Probit statistical analysis method.

Glyphosate determination
Samples were filtered through a Whatman 0.45 mm membrane (cellulose acetate). Glyphosate was derivatized by the addition of 0.25 mL of borate buffer 5% and 0.30 mL of FMOC-Cl (2 mmol/L) in $CHCl_3$ to 1 mL of water sample, at 40 °C, kept in the dark. The reaction was stopped after 24 h, by adding 0.30 mL H_3PO_4 (2%, Merck, Darmstadt, Germany), and kept refrigerated until analyzed. The derivatized product (Gly-FMOC) was analyzed by high performance liquid chromatography (HPLC) (CRB-6A; detector FLD, RF-10 AXL, Shimadzu, Kyoto, Japan) using a Supelco/Ascentis RP 18 Column (3 µm particle size, length 100 mm and I.D: 3 mm). The mobile phase used was acetonitrile: 0.05 M phosphate buffer (pH 6), with a gradient elution starting at 10% acetonitrile and progressing linearly to 40% acetonitrile, flow: 0.5 mL/min; fluorescence detection conditions were: excitation, 266 nm, emission, 315 nm. The injected sample volume was 20 µL. The chromatographic measurements were done at 40 °C. The mean recovery of the complete analytical technique was 79% ± 5% of glyphosate. Solvents used for pesticide analysis were from J.T. Baker (Avantor Performance Materials S.A., State of Mexico, Mexico). The detection limit was 0.05 mg/L.

Field experiment
The field work was performed at the Experimental Field Station of the School of Agronomic Science at La Plata University, located 8 km southwest of La Plata City, Buenos Aires, Argentina (35 01° S, 57 59° W). Soy was grown in an experimental field divided into 8 × 30 m plots. Irrigation sprinkler equipment was installed. It consisted of a perimeter pipe, 3 cm in diameter, provided with nine sprinkler heads mounted at a distance of 15 m from one another. Each impact sprinkler head was a Senninger 7025 model, 9.5 mm in diameter, providing a simulated rain of 16 mm/h with drops of 0.7-1 mm in diameter. The whole system was fed with water from a well, pumped with a 60,000 L/h pump. The field has a slope of 1%. At the lower end of each plot, a small trench was dug into the soil in order to capture the runoff water. A 5-liter bucket was buried in the trench.
The soy was seeded on 28 December 2009, with 45 seeds/m^2, and at a spacing of 35 cm between furrows. A single glyphosate application was made using a tractor-mounted sprayer when the crop had grown enough to attain complete soil cover (2 February 2010). Three plots were treated with glyphosate at a dose of 4 L/ha (2648 g active ingredient per ha). Four plots remained as controls without any application. The simulated rain episode was produced the day following the glyphosate application. It lasted until a surface runoff flux was observed, and stopped soon thereafter, in order to gather the whole runoff excess in the buried buckets. The runoff was transferred to dark bottles and immediately transported to the laboratory in coolers. The toxicity of the runoff water to the amphipod *H. curvispina* was assessed by means of laboratory toxicity tests. Three replicates from each plot were assessed. Procedures for toxicity tests to *H. curvispina* were the same as for the LC_{50} determinations. Soy leaves' toxicity to *H. curvispina* was also tested by adapted standardized protocols for soil toxicity test (USEPA 2000). Ten *H. curvispina* measuring 5-10 mm were exposed to a soy leaf in 150 mL of reconstituted moderately hard synthetic water (APHA 1998) kept in 250 mL beakers, in triplicate. Mortality was assessed after a 10-day exposure. The soy leaf was the only source of food for *H. curvispina*. Mortalities lower than 20% were considered as no effect.

Results

Table 1 summarizes the 48-h LC_{50} of glyphosate to *H. curvispina* determined on six independent occasions. The overall mean was 9.9 ± 1.7 mg/L. No mortality was observed in the controls.

Table 1. Acute toxicity of the Roundup Full II to the amphipod *Hyalella curvispina*

Point	May 2011	August 2011	November 2011	December 2011	January 2012	February 2012
	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim	Conc.(mg L^{-1}) 95% Conf. Lim
LC 1.0	4.2 (3.2–5.0)	5.1 (2.6–6.6)	3.8 (2–5.1)	3.8 (2.6–4.8)	4.0 (2.5–5.1)	5.4 (3.0-6.6)
LC 5.0	5.4 (4.3–6.1)	6.4 (3.8–7.9)	5.3 (3.4–6.8)	5.0 (3.8–6.2)	5.0 (3.4–5.9)	6.2(4–7.2)
LC 10.0	6.0 (5.0–6.8)	7.3 (4.8–8.6)	6.3 (4.4–7.8)	5.9 (4.6–6.9)	5.5 (4.1–6.5)	6.6 (4.6–7.6)
LC 15.0	6.5 (5.6–7.3)	7.8 (5.5–9.2)	7.2 (5.2–8.7)	6.6 (5.3–7.6)	6.0 (4.6–6.9)	7.0 (5.0–7.8)
LC 50.0	9.1(8.3–10)	11.1 (9.6–12.5)	12.2 (10.4–14)	10.2 (8.9–11.6)	8.2 (7.2–9.2)	8.6 (7.5–9.3)
LC 85.0	12.8 (11.6-14.8)	15.8 (13.8–20.6)	20.6 (17.4–27.2)	15.8 (13.7–19.5)	11.3 (10.1–13.8)	10.6 (9.8–12.5)
LC 90.0	13.9 (12.4–16.3)	17.1 (14.7–23.8)	23.3 (19.3–32.2)	17.6 (15–22.3)	12.2 (10.7-15.4)	11.2 (10.2–13.6)
LC 95.0	15.7(13.8–18.9)	19.3 (16.2–29.3)	28.0 (22.4-41.9)	20.6 (17.2–27.3)	13.7 (11.8–18.3)	12.1 (10.9–15.7)
LC 99.0	19.6 (16.6–25.1)	24.2 (19.0–43.8)	39.6 (29.4–68.9)	27.5 (21.8–40.1)	16.9 (13.8–25.4)	13.8 (12–20.5)
Slope ± SE	−1.9 ± 0.72	−2.29 ± 1.44	−0.29 ± 0.72	−0.79 ± 0.66	−2.06 ± 1.2	−5.4 ± 2.5
Intercept ± SE	5.6 ± 0.68	5.5 ± 1.28	3.6 ± 0.59	4.32 ± 0.58	5.98 ± 1.1	9.04 ± 2.5

In the present work, the researchers simulated the worst-case scenario by assessing the toxicity in runoff produced by a simulated rain event the day following a Roundup application in the soy plots. There was no *H. curvispina* mortality. Exposure of *H. curvispina* to soy leaves sampled immediately after the application did not produce mortality either.

Conclusion

Roundup Full II acute toxicity to *H. curvispina*, assayed in laboratory experiments, *H. curvispina* exposures to soy runoff and measured glyphosate concentrations in streams and runoff waters suggest low risk of acute toxicity to *H. curvispina* derived from Roundup Full II application in adjacent crops. Experiments reported in the present work refer to adults. Further studies are needed for juveniles, likely to be more sensitive. Being widely distributed and often attaining high densities in shallow South America water bodies, *H. curvispina* seems suitable for use as a sentinel organism for environmental impact assessment.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: A stuy which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). In addition, Roundup Full II® (66.2% glyphosate) is not the representative formulation for the glyphosate EU renewal, thus the paper is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The aim of this study was to assess the acute toxicity of Roundup Full II® (66.2% glyphosate), to Hyalella curvispina in laboratory and field assessments. The mean estimated 48-h LC50 of Roundup Full II® was 9.9 ± 1.7 mg/L.
RMS notes that the LC50 value seems low in comparison with the overall dataset available for aquatic invertebrates from regulatory studies. RMS also notes that the lowest LC1 was of 3.8 mg/L (lowest of 6 independent experiments) which may be assimilated to a NOEC, LC10 was 5.5 mg/L.
The low variability observed among 6 independent assays is indicative of the high reproducibility attained by H. curvispina toxicity testing with Roundup. The study design seems adequate and the results seems robust. However, no biological data are presented in the study report (only LCx values).

In a field experiment Roundup Full II® was applied to soybean plots. Simulated rain was generated the following day by means of irrigation sprinkler equipment. H. curvispina was exposed to runoff water and soy leaves. No mortality was observed.

The study states that further studies are needed for juveniles, likely to be more sensitive.

Roundup Full II was used. The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable. Therefore RMS considers that this study less relevant but supplementary (due to the different formulation tested) and reliable with restrictions.

B.9.2.5. Long-term and chronic toxicity to aquatic invertebrates

Data point:	CA 8.2.5.1/008
Report author	Levine, S.L. *et al.*
Report year	2015
Report title	Aminomethylphosphonic acid has low chronic toxicity to *Daphnia magna* and *Pimephales promelas*
Document No	DOI: 10.1002/etc.2940 E-ISSN: 1552-8618
Guidelines followed in study	OECD 211 (2008), OECD 210 (1992)
Deviations from current test guideline identified by the applicant: See RMS analysis in RMS comment box	*Deviations from current OECD guideline 211 (2012): None*
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Yes/Reliable

Full summary

The purpose of the present study was to assess the potential for chronic toxicity of AMPA to fathead minnow (*Pimephales promelas*) and *Daphnia magna*. Chronic toxicity to *P. promelas* was evaluated in a fish early–life stage study. The primary endpoints were larval survival, growth, and development. The chronic toxicity to *D. magna* was evaluated in a *Daphnia* reproduction test. The primary endpoints were survival, growth, and reproduction.

The NOAEC for *P. promelas* was determined to be 12 mg/L, the highest concentration tested. The no-observed-effect concentration for *D. magna* was determined to be 15 mg/L.

Materials and methods

Test substance

Synthesis of AMPA was performed by Chemir, and it had a purity of 98.7%. The water solubility for AMPA is reported to be 10 500 mg/L (based on glyphosate acid solubility data [RMS Germany. 2013]); therefore, solvent (to aid the dissolution of AMPA into water) was not required for the aquatic exposures. Stock solutions for waterborne exposures were prepared in well water, appeared clear and colorless after mixing, and were stored under refrigerated conditions (~ 4 ± 1 °C).

For the *D. magna* reproduction study, primary stocks and test solutions were prepared every 2 d to 3 d during the test. A primary stock solution was prepared in ultraviolet sterilized dilution water at a nominal concentration of 120 mg AMPA/L, equivalent to the highest concentration tested. Proportional dilutions of the primary stock solution were made in dilution water to prepare test solutions at nominal concentrations of 7.5 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, and 60 mg AMPA/L.

For the fish early–life stage study, stock solutions were delivered using syringe pumps into mixing vessels and mixed with diluent water in a continuous diluter system to prepare nominal test concentrations of 0.75 mg AMPA/L, 1.5 mg AMPA/L, 3.0 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L. Delivery of the test solutions was started 7 d prior to the initiation of the test to achieve equilibrium of the test substance in the test chambers.

Daphnia magna reproduction study—Culturing, exposure, and observations

Daphnia magna are the required cladoceran test species under the Organisation for Economic Co-operation and Development (OECD) 211 guideline [OECD 2008]. *Daphnia magna* was tested because it is representative of an important group of freshwater invertebrates and has a long and successful history as a test organism in the laboratory. Neonates (juveniles) <24 h old were used to initiate the test and were obtained from established cultures. Parental daphnids were cultured in well water that was filtered with a 0.45-µm filter and passed through an ultraviolet sterilizer. The source of well water was characterized as moderately hard water with an average specific conductance of 362 µS/cm, hardness of 132 mg/L as $CaCO_3$, alkalinity of 173 mg/L as $CaCO_3$, and pH of 8.2 during the 4-wk period immediately preceding the test.

During the 2-wk period preceding the test, culture temperatures ranged from 19.6 °C to 20.8 °C, pH from 8.1 to 8.7, and dissolved oxygen from 7.6 mg/L to 9.5 mg/L. During culturing and testing, daphnids were fed daily with a mixture of yeast, cereal grass medium, and trout chow, as well as a suspension of the freshwater green alga *Pseudokirchneriella subcapitata*. During the test, organisms in each test chamber were fed 0.5 mL of yeast–cereal–trout chow and 1.0 mL of algae, which represented 0.60 mg C/daphnid/d. Although this amount of feed exceeded the OECD guideline recommended amount of 0.1 mg C/daphnid/d to 0.2 mg C/daphnid/d, an excess amount was fed to maintain sufficient feed in the system to support acceptable reproduction rates, which is an acceptable deviation from the testing guideline.

The 4 adult daphnids used to supply neonates for the test were held for 19 d prior to collection of the juveniles for testing and had each produced at least 1 previous brood. Adult daphnids in the culture had produced an average of at least 3 young per adult per day over the 7-d period prior to the test. The adults showed no signs of disease or stress, and no ephippia were produced during the holding period. To initiate the test, juvenile daphnids were collected from the cultures and indiscriminately transferred 1 or 2 at a time into the transfer chambers that were impartially assigned to a control or treatment group until each transfer chamber contained 10 daphnids. All animals were released from the transfer chambers into the assigned test chambers below the water surface (to avoid air contact) using wide-bore pipettes to not

harm the neonates.

We tested AMPA in a semistatic renewal design with the renewal of test solutions every 2 d or 3 d. Concentrations of AMPA were measured on 3 occasions during the test: at the beginning and end of the first renewal cycle, at the beginning and end of the longest renewal cycle during the second wk of the test, and at the beginning and end of the last renewal cycle. Test chambers were 250-mL glass beakers that contained approximately 200 mL of test solution and were loosely covered with plastic Petri dishes. Beakers were impartially positioned in an environmental chamber that was programmed to maintain the target water temperature (20 ± 1 °C) throughout the test period. A 16:8-h light:dark photoperiod was used with a 30-min transition period of low light intensity when lights went on and off to avoid sudden changes in lighting. Lighting was provided by fluorescent light bulbs that emit wavelengths similar to natural sunlight. At test initiation the light intensity at the water surface of 1 representative test chamber was 296 lux (measured with a SPER Scientific Model 840006C light meter).

Temperature was measured continuously in 2 replicate test chambers in each treatment group, and measurements rotated among replicates in each group. Dissolved oxygen and pH were measured in the newly prepared solutions for each treatment group at test initiation and on renewal days and in the old solutions from 2 replicate test chambers in each treatment and control group on renewal days and at test termination. When a first-generation daphnid was found dead, measurements of temperature, dissolved oxygen, and pH were taken in the replicate at that time and then discontinued. Hardness, alkalinity, and specific conductance were measured in batch solutions of the negative control; the highest test concentration at test initiation and on 1 renewal day each week (day 7 and day 14); and pooled replicate solutions at test termination. Total organic carbon (TOC) was measured in the dilution water at test initiation and termination using a Shimadzu model TOC-VCSH analyzer and following the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association]. Hardness and alkalinity were measured by titration based on procedures in the *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association].

First-generation daphnids were observed daily during the test for immobility, the onset of reproduction, and clinical signs of toxicity. Following the onset of reproduction, the second- generation daphnids were counted 3 times per week and at test termination (day 21). Body lengths and dry weights of the surviving first-generation daphnids were measured at the end of the exposure period.

Fish early–life stage study—Culturing, exposure, and observations

Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas* with the exception of doubling the required level of replication [OECD 1992]. We selected *P. promelas* for the early–life stage study based on past use and ease of handling in the laboratory. Embryos (Chesapeake Cultures) were examined under a dissecting microscope to select healthy, viable specimens at approximately the same stage of development (<24 h). Embryos collected for use in the test were from 10 individual spawns and were <24 h old when the test initiated. Test chambers were 9-L glass aquaria filled with approximately 7 L of test solution and contained an embryo incubation cup attached to a reciprocating rocker arm (2 rpm) for water circulation during embryo incubation. To initiate the test, groups of 1 to 3 embryos were impartially distributed among incubation cups until each cup contained 20 embryos. A single incubation cup constructed from 50-mm-diameter glass cylinders with 425-µm nylon screen mesh attached to the bottom was placed into each test chamber. The incubation cup with the embryos was impartially assigned to each of the control and treatment groups.

The test was conducted in a temperature-controlled environmental chamber designed to maintain the target test temperature of 25 ± 1 °C throughout the test period. Temperature was measured in each test chamber at the beginning of the test, weekly during the test, and at the end of the test using a liquid-in-glass thermometer. Temperature also was monitored continuously in 1 negative control replicate using a Fulscope ER/C Recorder. Fluorescent light bulbs that emit wavelengths similar to natural sunlight were used on a 16:8-h light:dark photoperiod. A 30-min transition period of low light intensity was provided when lights went on and off to avoid sudden changes in lighting.

The negative (dilution water) control and AMPA test concentrations were delivered in a continuous-flow diluter. Syringe pumps (Harvard Apparatus) delivered the stock solutions (at a rate of 30 µL/min) into mixing chambers and mixed with dilution water (at a rate of 125 mL/min) to achieve the target test concentrations. The flow of dilution water to the mixing chambers was controlled by rotameters that were calibrated prior to test initiation and verified at weekly intervals during the test. The flow of test water from each mixing chamber was split and directed into 4 replicate test chambers. The proportion of the test water that was split into each replicate was checked prior to the test and at approximately weekly intervals during the test to ensure that flow rates varied by no more than ± 10% of the mean for the 4 replicates. The diluter flow rate was adjusted to provide approximately 6 volume additions of test water in each test chamber per day. The general operation of the diluter was checked visually at least 2 times/d during the test and at least once at the end of the test.

According to the OECD guideline, concentrations above the 96-h lethal concentration for 50% of the population or 10 mg/L, whichever is lower, need not be tested. To assure that a mean measured concentration ≥10 mg/L was tested, the highest nominal test concentration of 12 mg/L and the lower concentrations of 6 mg/L, 3 mg/L, 1.5 mg/L, and 0.75 mg/L were selected. Stock solutions were stored under refrigerated conditions, and fresh aliquots were placed in the syringe pumps daily during the test. Water samples were collected from 1 test chamber of each treatment and control group 4 d prior to test initiation to confirm the operation of the diluter. Water samples were collected from alternating replicate test chambers of each treatment and control group on day 0, day 7, day 14, day 21, day 28, and day 33 (test termination) to determine concentrations of the test substance in the test chambers. All samples were collected at mid-depth in the test chambers, placed in glass vials, and processed immediately for analysis.

Dissolved oxygen and pH were measured in alternating replicates of each treatment and control group at the beginning of the test, weekly during the test, and at the end of the test. Hardness, alkalinity, and specific conductance were measured in alternating replicates of the negative control (dilution water) and the highest concentration treatment group at the beginning of the test, weekly during the test and at the end of the test. Hardness and alkalinity were measured by titration based on procedures in *Standard Methods for the Examination of Water and Wastewater* [American Public Health Association], and specific conductance was measured using an Acorn Series Model CON6 Conductivity-Temperature meter.

During the first day of exposure, embryos were observed twice for mortality and fungal infection. Thereafter, until hatching was complete, observations of embryo mortality and the removal of dead embryos were performed once daily. When hatching reached >90% in the control groups on day 5 of the test, the larvae were released to their respective test chambers and the posthatch period began. During the 28-d posthatch exposure period, the larvae were observed daily to evaluate the mortality and the numbers of individuals exhibiting clinical signs of toxicity or abnormal behavior. From these observations, time to hatch, hatching success, and posthatch growth and survival were evaluated. Hatching success was calculated as the percentage of embryos that hatched successfully. Posthatch survival was calculated from the number of larvae that survived to test termination as a percentage of the number of embryos that hatched successfully.

Newly hatched larvae were fed live brine shrimp nauplii (*Artemia* sp.) 3 times/d during the first 7 d of the posthatch period. Thereafter, they were fed live brine shrimp nauplii 3 times/d on weekdays and at least 2 times/d on weekends. Fish were not fed for approximately 48 h prior to the termination of the test to allow for clearance of the digestive tract before weight measurements were made. To ensure that the feeding rate per fish remained constant, rations were adjusted at least weekly. The test chamber loading rate (the total wet wt of fish per liter of water in the tank) at the end of the test was 0.32 g fish/L.

Posthatch growth of *P. promelas* was evaluated at the conclusion of the 28-d posthatch exposure period. Total length for each surviving fish was measured to the nearest 1mm using a metric ruler, with wet and dry weights measured to the nearest 0.1 mg using an analytical balance. Fish were placed in an oven at 60 °C for up to approximately 48 h to obtain dry weight data.

Analytical method for detection of AMPA

Samples were diluted, as appropriate, with freshwater. The 2.0 mL of diluted sample and/or external calibration standards were placed into the 15-mL test tube. Then, 1.0 mL of 0.37 M aqueous potassium tetraborate was added to each test tube, followed by 2.0 mL of 0.025 M NBD-C1 (methanolic) for derivatization. Solutions were capped, mixed, and heated at approximately 80 °C for 40 min. Next, 1.0 mL of 1.2 M HCl was added to each test tube, and samples were then left undisturbed for approximately 10 min prior to analysis. Samples (25 µL injection volume) were analyzed on an Agilent Series 1100/1200 high performance liquid chromatograph equipped with an Agilent Series 1100 variable wavelength detector at 500 nm. Chromatographic separations were achieved using a YMC-Pack ODS-AM (150 mm × 4.6 mm, 3 µm particle size) analytical column at a temperature of 40 °C and eluted over a gradient of 0.1% H_3PO_4 (solvent A) and CH_3CN (solvent B). The retention time for AMPA was approximately 6.5 min to 7.3 min, and the method limit of quantitation for these analyses was defined as 0.4 mg AMPA/L.

Statistical and power analyses

Test endpoints analyzed statistically in the *Daphnia* test for first-generation daphnids were survival, reproduction (the number of live young produced per 21-d surviving adult), and growth (length and dry wt). Neonates produced by those first-generation daphnids that did not survive the full 21 d were excluded from analysis of reproduction.

Test endpoints analyzed statistically in the fish early–life stage test were hatching success, larval survival, and growth (total length, wet wt, and dry wt). Data on time to hatch were evaluated by visual interpretation.

Discrete-variables data were analyzed using Fisher's exact test to identify treatment groups that showed a statistically significant difference ($p \leq 0.050$) from the negative control. All continuous-variable data were evaluated for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test ($p = 0.010$). When the data passed the assumptions of normality and homogeneity of variance, those treatments that were significantly different from the control means were identified using the 1-tailed Dunnett's test ($p \leq 0.050$). All statistical tests were performed using a personal computer with SAS software. The results of the statistical analyses were used to aid in the determination of the no-observed–adverse effect concentration (NOAEC), defined as the greatest test concentration that produced no significant treatment-related adverse effects on survival, reproduction, or growth.

Results

Daphnia magna survival, growth, and reproduction

Water temperatures were maintained within the targeted range of 20 ± 1 °C, dissolved oxygen concentrations remained ≥76% of saturation (6.8 mg/L), and pH ranged from 7.1 to 8.6 during the test. Specific conductance, hardness, and alkalinity were similar between the control and treatment groups and did not appear to be influenced by AMPA. The TOC in the dilution water at test initiation and termination was <1 mg C/L.

Table 8.2.5-1: Means and ranges of water quality measurements taken during the 21-Day *D. magna* exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± Std. Dev. and Range of Measured Parameters					
	Temperature (°C)	Dissolved Oxygen [1] (mg/L)	pH	Hardness [2] (mg/L as $CaCO_3$)	Alkalinity [2] (mg/L as $CaCO_3$)	Conductivity [4] (µS/cm)
Negative Control	19.8 ± 0.55 (19.1 – 20.8)	8.2 ± 0.58 (7.2 – 9.1)	8.4 ± 0.10 (8.3 – 8.6)	135 ± 2 (132 - 136)	167 ± 5 (160 – 171)	339 ± 46 (280 – 391)
7.4	19.9 ± 0.48 (19.2 – 20.7)	8.0 ± 0.60 (7.1 – 9.1)	8.4 ± 0.09 (8.3 – 8.6)	--	--	--
15	19.9 ± 0.50 (19.1 – 20.8)	7.9 ± 0.65 (6.9 – 9.1)	8.4 ± 0.08 (8.2 – 8.6)	--	--	--
30	19.9 ± 0.54 (19.0 – 20.7)	7.9 ± 0.66 (6.8 – 9.1)	8.3 ± 0.10 (8.2 – 8.5)	--	--	--
57	20.0 ± 0.52 (19.1 – 20.6)	7.9 ± 0.60 (7.0 – 9.1)	8.2 ± 0.23 (7.7 – 8.5)	--	--	--
120	20.1 ± 0.54 (19.1 – 20.7)	8.0 ± 0.54 (7.1 – 9.1)	7.9 ± 0.48 (7.1 – 8.4)	139 ± 2 (136 – 140)	164 ± 6 (156 – 170)	340 ± 46 (274 – 381)

[1] A dissolved oxygen concentration of 9.1 mg/L represents 100% saturation at 20°C in freshwater. Any recorded dissolved oxygen measurement greater than 100% saturation is reported as 9.1 mg/L.
[2] -- = no measurements scheduled.

Measured concentrations of AMPA for the *D. magna* study were close to nominal concentrations throughout the renewal periods. Concentrations of AMPA in the new test solutions prepared and sampled on day 0, day 9, and day 19 ranged from 92.5% to 106% of the nominal concentrations. Concentrations of AMPA in the old test solutions sampled immediately prior to renewal on day 2, day 12, and at test termination on day 21 ranged from 78.6% to 117% of the nominal concentrations. When the measured concentrations of the samples collected during the test were averaged for each treatment group, the mean measured test concentrations were 7.4 mg AMPA/L, 15 mg AMPA/L, 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L.

There was no significant effect of AMPA on individually exposed first-generation daphnids across the treatments, and survival was ≥80%. A summary of adult survival is presented in Figure A below. After 21 d of exposure, survival in the negative control, 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L groups was 75%, 80%, 100%, 70%, 100%, and 90%, respectively. Although survival in the negative control group was slightly below the 80% criterion in OECD guideline 211, it is not considered to have impacted the validity of the present study because of the small difference and with the final mortality occurring near the end of the study. In addition, there was ≥80% survival in all treatment groups. The surviving daphnids in the control replicates appeared normal and healthy through the end of the test, indicating that the mortality observed was attributed to incidental death and not the health of the organisms. In addition, the percentage survival of the control replicates was within the control criterion of 70% as specified in the American Society for Testing and Materials standard guide E 1193-97 [ASTM International. 1997]. Survival in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups at test termination did not follow a concentration–response pattern and was 80%, 100%, 70%, 100%, and 90%, respectively. No significant differences in survival were detected in any of the AMPA treatment groups in comparison with the control ($p > 0.05$, Fisher's exact test). Consequently, the no-observed-effect concentration (NOEC) for survival was 120 mg AMPA/L.

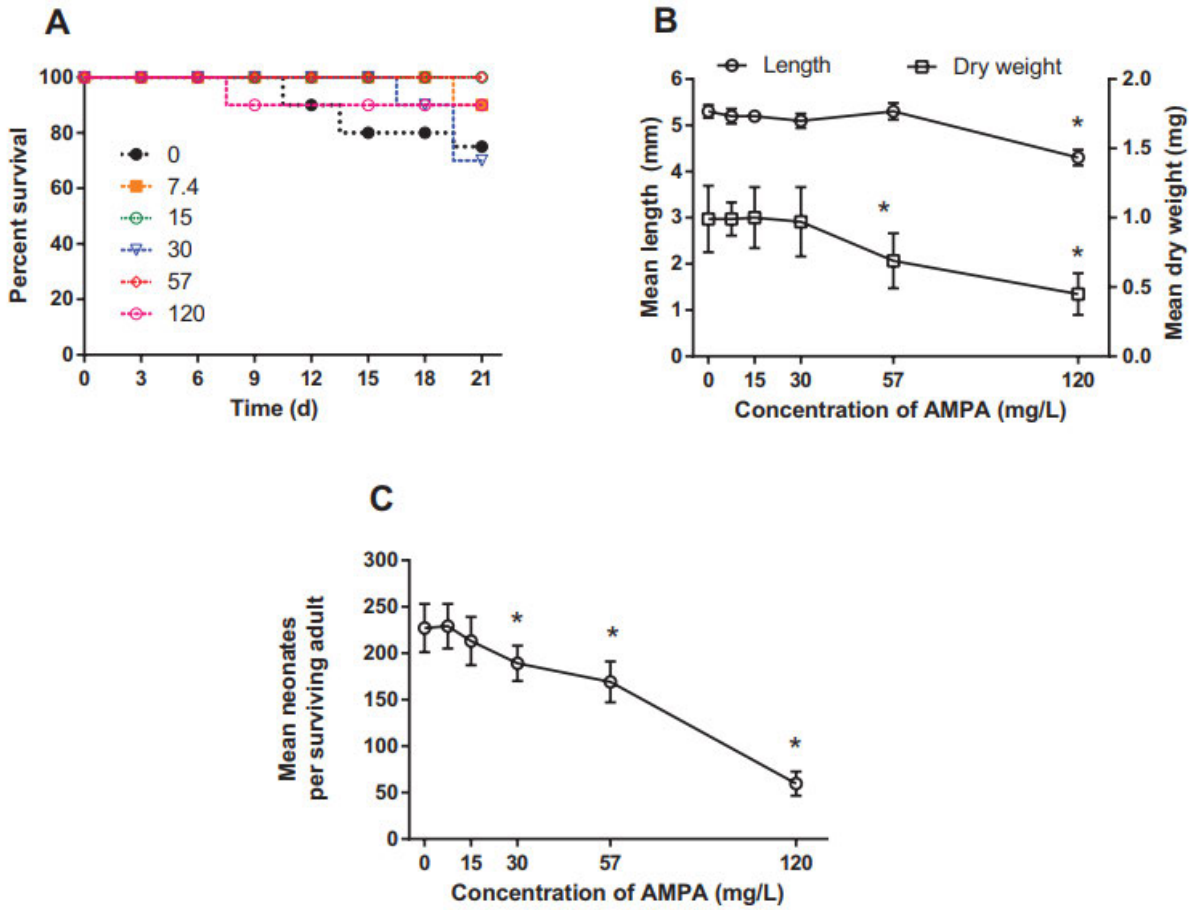


Figure 8.2.5-1: (A) Survival (percentage) of *Daphnia magna* exposed to increasing concentrations of AMPA for 21 d. (B) Sublethal endpoints in 21-d chronic *D. magna*: body length and dry weight. (C) Reproductive endpoint neonates per surviving adults. * Statistically significant difference ($p < 0.05$) from the control (0 mg/L).

Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups that survived until test termination generally appeared normal. In the 120–mg AMPA/L treatment group, all surviving first-generation daphnids appeared pale in comparison with the control organisms from day 5 through the end of the test. Daphnids in this treatment group were also observed to be smaller than the control organisms from day 7 through the end of the test. All surviving daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, and 57–mg AMPA/L treatment groups were normal in appearance throughout the test and at test termination, with the exception of 1 daphnid in the 57–mg AMPA/L treatment group that appeared pale on day 6 of the test but appeared normal from day 7 to the end of the test.

A summary of production of neonates by surviving first-generation daphnids is presented in Figure C above. The first day of brood production in the negative controls and in all AMPA treatment replicates was day 7, day 8, or day 9 of the test, indicating that there was no apparent delay in the onset of production at any concentration of AMPA tested. Immobile neonates were noted in the control, 7.4–mg AMPA/L, and 57–mg AMPA/L treatment groups. However, the mean number of immobile neonates per surviving adult in these replicates was less than 1. No aborted brood or aborted eggs were present in the control or any of the AMPA treatment replicates. No males or ephippia were produced during the test.

Summaries of the mean lengths and dry weights of surviving first-generation daphnids are presented in Figure 8.2.5-2 'B' below. Daphnids in the negative control group averaged 5.3 mm in length and 0.99 mg in dry weight. Daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups had mean lengths of 5.2 mm, 5.2 mm, 5.1 mm, 5.3 mm, and 4.3 mm, respectively, and mean dry weights of 0.99 mg, 1.0 mg, 0.97 mg, 0.69 mg, and 0.45 mg, respectively. There were significant decreases in length in the 30–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$) but not in the 57–mg AMPA/L treatment group. There were significant decreases in dry weight in the 57–mg AMPA/L and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOEC for growth was 30 mg AMPA/L.

Adult daphnids in the negative control group produced an average of 227 live young per surviving adult (coefficient of variance of 11.6%), well above the validity criterion of ≥60 live young per surviving adult. Adult daphnids in the 7.4–mg AMPA/L, 15–mg AMPA/L, 30–mg AMPA/L, 57–mg AMPA/ L, and 120–mg AMPA/L treatment groups produced an average of 229, 213, 189, 169, and 59.6 live young per surviving adult, respectively. There was a significant decrease in mean neonate production in the 30–mg AMPA/L, 57–mg AMPA/L, and 120–mg AMPA/L treatment groups in comparison with the negative control ($p \leq 0.05$). Consequently, the NOAEC for reproduction is 15 mg AMPA/L.

Pimephales promelas embryo hatching success, growth, and survival

Samples of the test solutions collected during the test had measured concentrations that ranged from 82.5% to 117% of nominal concentrations. When the measured concentrations of test solution samples collected on day 0, day 7, day 14, day 21, day 28, and day 33 of the test were averaged for each treatment group, the mean measured test concentrations were 0.73 mg AMPA/L, 1.5 mg AMPA/L, 2.9 mg AMPA/L, 6.0 mg AMPA/L, and 12 mg AMPA/L, which represented 97%, 100%, 97%, 100%, and 100% of nominal concentrations, respectively. Therefore, the results of the present study have been based on mean measured concentrations. The analytical results are summarized in Supplemental Data, Table S4.

Hatching success of the *P. promelas* embryos is summarized in Figure 8.2.5-2'A' below. Daily observations of the embryos indicated that there were no apparent differences in time to hatch between the negative control group and any of the AMPA treatment groups. All *P. promelas* embryos in the control and treatment replicates hatched by day 5 of the test. Hatching reached >90% in the control groups on day 5 of the test, at which time the larvae were released to their respective test chambers.

Hatching success in the negative control, 0.73–mg active ingredient (a.i.)/L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 99%, 100%, 100%, 100%, 100%, and 99%, respectively (Figure A below). There were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Larval survival in the negative control, 0.73–mg a.i./L, 1.5–mg a.i./L, 2.9–mg a.i./L, 6.0–mg a.i./L, and 12–mg a.i./L treatment groups was 91%, 91%, 93%, 90%, 91%, and 92% (Figure A below), respectively; and there were no statistically significant differences in hatching success in any of the AMPA treatment groups in comparison with the negative control ($p > 0.05$). In addition, there were no statistically significant reductions in total length, wet weight, and dry weight (Figure B below) among fish in the AMPA treatment groups in comparison with the negative control ($p > 0.05$). Based on an evaluation of each of

these endpoints, the NOAEC for growth was 12 mg a.i./L.

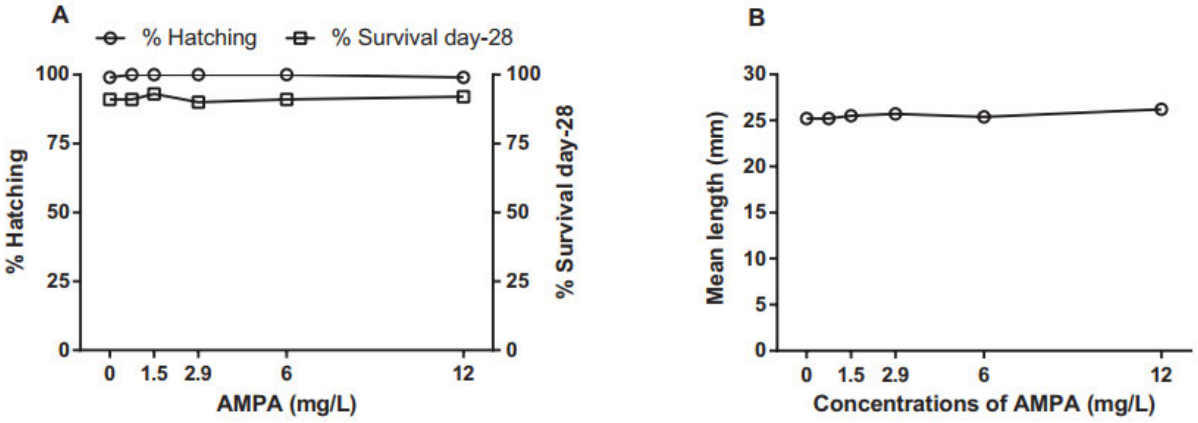


Figure 8.2.5-2: (A) Hatching success (percentage) and survival (percentage) at day 28 of *Pimephales promelas* in an early–life stage study with aminomethylphosphonic acid (AMPA). (B) Body length (millimeters) of *P. promelas* exposed to AMPA.

Table 8.2.5-2. Means and Ranges of Water Quality Measurements Taken During the 33-Day *P. promelas* Exposure to AMPA

Mean Measured Concentration (mg AMPA/L)	Mean ± SD and Range of Measured Parameters					
	Temperature (°C)	DO[2] (mg/L)	pH	Hardness[2] (mg/L as $CaCO_3$)	Alkalinity[2] (mg/L as $CaCO_3$)	Conductivity[2] (µS/cm)
Assay Control	24.8 ± 0.27 (24.4 – 25.4)	7.9 ± 0.40 (7.4 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	136 ± 3 (132 – 140)	172 ± 5 (166 – 178)	378 ± 11 (361 – 393)
0.73	24.7 ± 0.59 (23.7 – 25.5)	7.9 ± 0.39 (7.4 – 8.2)	8.1 ± 0.09 (8.0 – 8.2)	-- --	-- --	-- --
1.5	24.9 ± 0.66 (23.9 – 25.7)	7.9 ± 0.39 (7.3 – 8.2)	8.1 ± 0.10 (8.0 – 8.2)	-- --	-- --	-- --
2.9	25.0 ± 0.52 (24.2 – 25.6)	7.9 ± 0.33 (7.5 – 8.2)	8.1 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
6.0	25.0 ± 0.46 (24.3 – 25.7)	7.9 ± 0.34 (7.5 – 8.2)	8.0 ± 0.12 (7.9 – 8.2)	-- --	-- --	-- --
12	25.1 ± 0.48 (24.3 – 25.7)	7.9 ± 0.37 (7.4 – 8.2)	7.9 ± 0.13 (7.8 – 8.1)	138 ± 2 (136 – 140)	174 ± 4 (170 – 180)	379 ± 11 (365 – 395)

[1] A dissolved oxygen concentration of 4.9 mg/L represents 60% saturation at 25°C in freshwater. Any recorded DO measurement greater than 100% saturation is reported as 8.2 mg/L.
[2] -- = no measurements scheduled.

Conclusion

For *D. magna* exposed to concentrations ranging from 7.4 mg AMPA/L to 120 mg AMPA/L for 21 d, reproduction was the most sensitive endpoint with significant treatment-related effects noted at 30 mg AMPA/L, 57 mg AMPA/L, and 120 mg AMPA/L. Consequently, the NOAEC based on reproduction was 15 mg AMPA/L. No impact was noted on hatching success, survival, or growth in *P. promelas* embryos exposed to concentrations ranging from 0.73 mg AMPA/L to 12 mg AMPA/L for 33 d. Consequently, the NOAEC was 12 mg AMPA/L, the greatest concentration tested. These values exceed the worst-case water concentrations from conservative modelling and surface water monitoring data by 2 to 3 orders of magnitude, indicating no unacceptable chronic risk for vertebrate and invertebrate aquatic organisms from environ mental exposure to AMPA.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Chronic toxicity tests of the glyphosate environmental metabolite aminomethylphosphonic acid (AMPA) were performed with fathead minnow (*Pimephales promelas*) and *Daphnia magna*.
During a 21-d exposure period under semi-static test conditions the effects on survival, growth, and reproduction of the cladoceran *Daphnia magna* were determined resulting in a no-observed-effect concentration (NOEC) of 15 mg AMPA/L. During a 33-d exposure period under continuous renewal test conditions the effects on time to hatch, hatching success, posthatch growth and survival of the fish *Pimephales promelas* were assessed resulting in an NOAEC of 12 mg AMPA/L, the highest tested concentration. Test methodology followed the procedure outlined in the OECD 210 test guideline for *P. promelas*. For the chronic test on *Daphnia magna* the OECD 211 guideline is mentioned in the full text.

The study is well documented and all relevant information, e.g. information on the test item, test design, application method and implementation of the study, is available. In addition, a chemical analysis of test solutions was performed. All information for evaluation of the study is given. The study is considered as reliable.

Assessment and conclusion by RMS:
The daphnid part of this publication actually corresponds to the study summarized above and already assessed by RMS (CA 8.2.5.1/007, [redacted] et al. 2011, AMPA (Aminomethylphosphonic acid): A semi-static life cycle toxicity test with the Cladoceran (*Daphnia magna*), 139A-393).

The fish part of this publication actually corresponds to the study summarized above and already assessed by RMS (CA 8.2.2.1/004, [redacted] et al., 2011, AMPA (Aminomethylphosphonic acid): An early life-stage toxicity test with the fathead minnow (Pimephales promelas), 139A-39A).

Therefore, this publication was not assessed by RMS.

B.9.2.6. Effects on algal growth

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B.9.2.7. Effects on aquatic macrophytes

B.9.2.7.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.2.7/013
Report author	Yanhui, T.. *et al.*
Report year	2015
Report title	Growth inhibition of two herbicides on *Spirodela polyrhiza*
Document No	ISSN: 1002-5480
Guidelines followed in study	OECD 221
Deviations from current test guideline	Not reported
Previous evaluation	No

GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability (RMS):	Applicant : Yes/Reliable with restrictions RMS : Relevant / reliability not assignable (data gap : provide an English certified translation)

Summary

The inhibitory activities of glyphosate on the aquatic macrophyte *Spirodela polyrhiza*, were studied in the laboratory by using quantity of the thallus as test indicator. The effects of glyphosate were tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza*, and the inhibitory rate increased with higher concentrations. The 168 hour-EC_{50} value was determined to be 12.817 mg/L.

Materials and Methods

Test materials and culture

The tested organism, *Spirodela polyrhiza*, was introduced from Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

The *Spirodela polyrhiza* was placed in a crystal dish with the volume of 300 mL (10 × 5cm). The Swiss standard (SIS) culture medium was added (see table below). The light was 9000 - 10 000lx incandescent light, the temperature was 24 ± 2℃, and the culture medium was replaced every 7 days to maintain the stability of the concentration of the nutritional ingredients in the solution. It can only be used in the experiment after 14 days of continuous pre-culture. Before the experiment, enough 4-leaf *Spirodela polyrhiza* with good shape and similar shape and size were selected to carry out the experiment. The above experimental operations should be carried out in an ultra-clean work table to prevent culture medium pollution.

Main instruments and test reagents for the test

Main instruments and reagents

Intelligent artificial climate box PRX-350B (Ningbo Saifu Experimental instrument Co., Ltd.), super clean worktable VS-1300L-U (Sujing Antai), biosafety cabinet BHC-1300 II A/B3 (Suzhou Antai); Glyphosate 96.8% original drug (provided by Ministry of Agriculture Pesticide Inspection Institute), Dimethylformamide (Analytical Reagent, Beijing Chemical Plant), Twin 80 (Analytical Reagent, Beijing Chemical Plant).

Table B.9.2.7-1: (SIS) culture medium component*

Storage solution serial No.	Reagent	Storage solution concentration (g/L)	Concentration of culture medium (mg/L)
A	$NaNO_3$	8.5	85
	KH_2PO_4	1.34	13.4
B	$MgSO_4\ 7H_2O$	15	75
C	$CaCl_2{\cdot}2H_2O$	7.2	36
D	Na_2CO_3	4	20
E	$Na_2EDTA{\cdot}2H_2O$	0.28	1.4
	$FeCl_3{\cdot}6H_2O$	0.17	0.84
F	H_3BO_3	1	1
	$CuSO_4\ 5H_2O$	0.005	0.005
	$ZnSO_4\ 7H_2O$	0.05	0.05
	$MnCl_2\ 4H_2O$	0.2	0.2
	$Na_2MoO_4\ 2H_2O$	0.01	0.01
	$Co(NO_3)_2\ 6H_2O$	0.01	0.01

* All storage solutions shall be kept in refrigerated and dark conditions, and the storage solution AE can be kept for 6 months, while the reserve liquid F can only be kept for 1 month. Prepare 1LSIS medium, take 10 mL stock solution A, 5 mL storage solution B~E, 1 mL stock solution F into volumetric flask, add 900 mL distilled water, adjust pH to 6~7 with 1 mol/L HCl, and then use distilled water to 1L.

Experimental Methods

Allocation of test mother liquid: 0.1 g glyphosate was obtained by dissolving it in aseptic water, and the volume was fixed to 100 mL capacity bottle. And then 1 000 mg/L glyphosate mother liquid could be obtained. After sealing the above liquid with sealing film, put it in the refrigerator at 4℃ for further test.

Experimental Design

On the basis of the pre-test, a series of concentration gradients are set according to the equal ratio difference. The concentrations of glyphosate were 8.4, 10.08, 12.096, 14.515, 17.418, 20.902 mg/L and solvent control group and blank control group. 200 mL (height > 2cm) culture solution containing different concentrations of glyphosate was added to the crystal dish with diameter 10 cm. Three selected *Spirodela polyrhizas* were put into the above toxic solution, sealed with an aseptic culture container ligated with a rubber band. 3 repeats were set up in each treatment, and finally they were randomly placed in an artificial climate box. The experimental conditions were consistent with the pre-culture conditions. In order to maintain the concentration of the test solution, semi-static culture was used in this experiment. PH was measured before replacing the culture test solution on the 3rd and 5th day, respectively. All the above operations should be operated under aseptic conditions to prevent culture medium pollution. The test period was seven days. After the experiment was over, the average specific growth rate μ of the blank control was calculated, and the growth inhibition percentage of each treatment group was also calculated.

Test Index

The number and growth condition of *Spirodela polyrhiza* in each treatment group were recorded every 2 days, and whether the culture medium was normal or not was also recorded. All clearly visible leaves should be counted. The increase of the number of *Spirodela polyrhiza* leaves indicated its growth, and the difference between each concentration group and the control group indicated the toxic effect.

Data Processing

The average specific growth rate (μ)

The average specific growth rate in a specific period is to calculate the growth variables (leaf number, total leaf area) during the logarithmic growth period, and the following formula is used to calculate each repetition of the control and treatment.

$I = \frac{\mu_c - \mu_t}{\mu_c} \times 100\%$

In this: I - Average specific growth inhibition rate, %;

μ_c - control group μ mean value;

μ_t - control group μ mean value

Results

In the process of effectiveness analysis, the solvent control group grew well and the solvent content was less than 100 μL/L; In addition, the pH variation range (0.6 - 1.2) was not more than 1.5 before and after the replacement of the *Spirodela polyrhiza* culture solution. The average specific growth rates of leaf bodies in each blank treatment group were calculated to be 0.294 d^{-1} and 0.317 d^{-1}, respectively, both > 0.275 d^{-1}. The average specific growth rate of leaves in each blank treatment group was 0.294 d^{-1} and 0.317 d^{-1}, respectively. The above test results meet the requirements of *Spirodela polyrhiza* growth inhibition test in OECD, and the test system is effective.

Effect of glyphosate on the growth of *Spirodela polyrhiza* can be seen from the Figure below. Within a certain range, the herbicide can inhibit the growth of *Spirodela polyrhiza*, and with the increase of the concentration of the test solutions, the inhibition effect is strengthened.

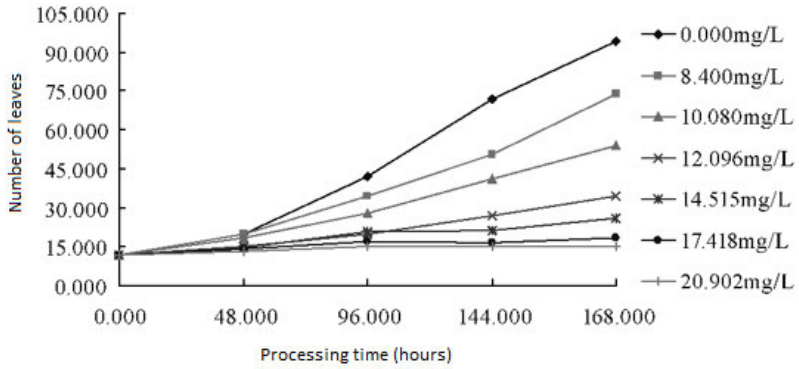


Figure B.9.2.7-1: Inhibition of different concentrations of glyphosate on the growth of *Spirodela polyrhiza*

The growth inhibition rates of glyphosate on the leaves of *Spirodela polyrhiza* can be seen from the table below. The coefficient of variation of each treatment group changed a bit, and the growth inhibition rates on the leaves of *Spirodela polyrhiza* showed significant differences at different concentrations of the test solution. Within a certain range, the growth inhibition rates on the leaves of the *Spirodela polyrhiza* increased with the increase of the concentration of the test solution.

Table B.9.2.7-2: Inhibition rate of different concentrations of glyphosate on the growth of Spirodela polyrhiza

Treatment concentration (mg/L)	Coefficient of variation (%)	Inhibition rate of growth*I (%)
0.000	1.359	0.000 ± 0.231g
8.400	2.707	11.650 ± 0.406f
10.080	1.231	26.926 ± 0.153e
12.096	5.600	48.512 ± 0.489d
14.515	4.980	62.456 ± 0.317c
17.418	7.070	78.548 ± 0.257b
20.902	15.230	88.113 ± 0.307a

* indicates growth inhibition rate ± standard error. In the same column of data, the same letter indicates that there is no significant difference at 0.05 level (P = 0.05).

The EC_{50} of glyphosate on the leaves of duckweed was calculated by using "SPSS Statistics 17.0" software. The EC_{50}, 95% confidence interval and linear equation of glyphosate for *Spirodela polyrhiza* were calculated (see table below). It can be seen from the correlation coefficient of the linear equation that the growth inhibition rate of the two herbicides on the *Spirodela polyrhiza* is a good linear relationship with the concentration of the test solution. The EC_{50} of glyphosate to the *Spirodela polyrhiza* was 12.817 mg/L.

Table B.9.2.7-3: Inhibitory medium concentration of glyphosate

Test solution	EC_{50} (mg/L)	EC_{50} 95% confidence interval	Linear equation
Glyphosate	12.817	12.256 - 13.388	$y = 5.928x – 6.567$ $R^2 = 0.993$

Conclusion

The results showed that glyphosate had remarkable effects on the growth inhibition of *Spirodela polyrhiza*, and the inhibitory rate increased with higher concentrations. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

III. CONCLUSIONS

Assessment and conclusion by applicant:

The effects of glyphosate to the aquatic macrophyte *Spirodela polyrhiza* was tested in a semi-static exposure of 7 days at concentrations between 8.4 and 20.902 mg/L. The 7 day-EC_{50} value was determined to be 12.817 mg/L.

This study was conducted to guideline but not to GLP. The test concentrations were not analytically verified and thus the exact exposure concentrations of the aquatic macrophyte are unknown. Therefore, the study should considered as reliable with restrictions.

Assessment and conclusion by RMS:

The study report is in Chinese and a translated version was not available to RMS.
Validity criteria, biomass and growth rates could not be checked as no raw data is presented and only the graphics or tables presented above are available in the study summary.
In addition, the study is not GLP and analytical measurements of nominal concentrations were not conducted.

Therefore, RMS considers this study is relevant but its reliability can not be assigned in absence of an English certified translation.

B.9.2.7.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Reno U. *et al.*
Report year	2014
Report title	The impact of Eskoba®, a glyphosate formulation, on the freshwater plankton community.
Document No	Water environment research (2014), Vol. 86, No. 12, pp. 2294-300
Guidelines followed in study	partially in accordance with OECD Guideline No. 201.
Deviations from current test guideline	Not mentioned
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

This study analyzed the acute effects of a glyphosate based herbicide (Eskoba®) on the microalgae *Chlorella vulgaris*, the cladoceran *Simocephalus vetulus*, and the copepod *Notodiaptomus conifer*, and evaluated the recovery ability of the surviving microcrustaceans. Survival, age of first reproduction, and fecundity were used as endpoints for *S. vetulus*, while survival and time to reach the adult stage were used as endpoints for *N. conifer*. The registered order of sensitivity was *S. vetulus* (48-hour effective concentration [EC_{50}]: 21 mg/L) > *C. vulgaris* (72-hour EC_{50}: 58.59 mg/ L) > *N. conifer* (48-hour EC_{50}: 95 mg/L). Despite the growth of *C. vulgaris* stimulated after 24 hours of exposure to the commercial formulation of glyphosate Eskoba®, it was inhibited after 48 hours by all the concentrations tested. In post-exposure experiments, microcrustaceans reduced their life expectancy, *S. vetulus* decreased its fertility, and *N. conifer* inhibited its sexual maturity.

Materials and Methods

Chemicals
The commercial formulation of glyphosate Eskoba® used in this study is composed of 48% (w/v) isopropylamine glyphosate and 52% inert ingredients and coadjuvants. The stock solution was prepared by diluting the commercial formulation of technical grade (95%) provided by Ciagro SRL (Santa Fe, Argentina) in sterile, bidistilled water to obtain a concentration of 1000-mg/L acid equivalent. It was kept in darkness at -4 °C until its analytical determination using a Dionex DX-100 ion chromatograph equipped with a conductivity detector, a suppressor, a column, and a precolumn. The eluent was NaOH

3.2 mM/Na_2CO_3 7.2 mM. The measured GLY concentration was 1067.5 (± 38.48) mg/L; this stock solution was used to prepare each test concentration for the experiments with the three species.

Test Organisms and Culture Conditions
The strain of *C. vulgaris* (CLV2) was cultivated using Bold Basal Medium (BBM) (Sager and Granik, 1953). The culture was maintained at constant temperature (23 ±1 °C) with uniform and continuous aeration, constant light intensity (approximately 8000 Lux), and continuous stirring using a 100-rpm magnetic plate.

Microcrustaceans
Simocephalus vetulus and *Notodiaptomus conifer* specimens were collected with a plankton net (100 µm) from lentic unpolluted waterbodies of the Parana River's alluvial valley. After being collected, the animals were carried to the laboratory for acclimation. Cladocerans and copepods were raised in different culture media because of their different nutritional requirements. Cladocerans were individually maintained in glass beakers with 30 mL of synthetic medium (APHA et al., 1998) comprising 2.4 g $MgSO_4$, 3.84 g $NaHCO_3$, 0.16 g KCl, and 2.4 g $CaSO_4$ * $2H_2O$ dissolved in 20 L of distilled water. Copepods were maintained in dechlorinated tap water, the physicochemical characteristics of which were similar to that of the Parana River, as follows: nitrates, < 0.1 mg/L; nitrites, 0.01 mg/L; ammonium, 0.29 mg/L NH_3; chlorides, 3.5 mg/L; sulphates, 8.3 mg/L; total alkalinity, 77 mg/L $CaCO_3$; bicarbonates, 94 mg/L; sodium, 7.7 mg/L; magnesium, 6.8 mg/L; calcium, 12.9 mg/L; potassium, 1.8 mg/L; chemical oxygen demand, 10 mg/L; and biological oxygen demand, 0.08 mg/L. Animals were fed three times a week with a drop of suspension of algae (*C. vulgaris*; absorbance = 1.5 λ, 650 nm) for each culture chamber. The water quality parameters, which were maintained constant, were as follows: pH 7.6 to 7.75; dissolved oxygen, 7.32 to 7.89 mg/L; temperature, 25 ± 2 °C; and photoperiod 16:8 (light:darkness).

Toxicity of Glyphosate (N-(phosphonomethyl)glycine) to C. vulgaris
The experimental treatments of algal growth inhibition with *C. vulgaris* were carried out according to the standard protocol of the Organisation for Economic Co-Operation and Development (1984), that is, microalgae were harvested in the exponential growth phase and then centrifuged and resuspended in sterile, ultrapure water. All the experiments started with the same initial cell density: 10^4 cells/mL. The algal density estimation was carried out directly using a Neubauer chamber (1.02 x 10^6 cells/mL) and, indirectly, by spectrophotometry (Abs 1.5 k; 650 nm). Control and treatments were triplicated; they were conducted in 100 mL of BBM medium in the following GLY concentrations: 0.5, 1, 2, 4, 8, and 16 mg/L. The controls were carried out in BBM medium without GLY.

Three replicates of 100 µL each were taken at 24, 48, and 72 hours for cells counting with a 400 x Olympus light microscope in a Neubauer chamber. In all instances, at least 25 squares were counted to ensure errors lower than 10% (Venrick, 1978). The considered endpoints were the effective concentration (EC_{50}) (72-hour EC_{50}) and the growth rate (µ). The 72-hour EC_{50} was estimated using the linear interpolation method (U.S. EPA, 2002). Differences between control and treatments ($\log_{10}$ [x] transformed values) were analyzed using repeated measures analysis of variation (ANOVA) ($\alpha = 0.05$).

Toxicity of (N-(phosphonomethyl)glycine) to Microcrustaceans
For microcrustaceans, acute (48-hour) toxicity assays were started with neonates (< 24 hours) of *S. vetulus* (U.S. EPA, 2002) and with copepodites of *N. conifer* at the fifth instar (Copepodite 5). Five GLY concentrations plus the control (without GLY) were used for each species: 3.2, 6.4, 12.8, 25.6 and 51.2 mg/L for *S. vetulus* and 20, 40, 80, 160, and 320 mg/L for *N. conifer*. Culture conditions were identical to those used for the stock culture. The number of replicates for *S. vetulus* and *N. conifer* assays were 30 and 20, respectively, per each concentration tested, placing one specimen per replica. As indicative of the toxic effect, the authors considered the complete immobilization of the organisms and the absence of response after to be stimulated or prodded by a metal rod. At 48 hours, the number of live and dead organisms was recorded. Results were considered acceptable when mortality in the control group was ≤10%. Levels of pH and oxygen were recorded during all the experiments and ranged from 7.6 to 8 mg/L and 6 to 8 mg/L, respectively. The lethal concentration 50 (LC_{50}) (e.g., the dose required

to kill half the members of the population tested) was determined at 48 hours; the 48-hour LC_{50} values and their 95% confidence limits were estimated using Probit analysis (Finney, 1971).
To evaluate the resilience (recovering capacity after the toxicant exposure) of both microcrustaceans, survivors of acute trials were moved to new containers with their respective culture media without GLY for 15 days under identical conditions to those used for the stock culture. Animals were fed three times a week with a drop of a suspension of *C. vulgaris* per chamber (absorbance = 1.5 λ, 650 nm). Both pH values and oxygen concentrations were recorded at the beginning and end of each assay, within the limits established by *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 1998). As endpoints for *S. vetulus*, mortality, age of first reproduction and fecundity (neonates/female) were considered. For *N. conifer*, the mortality and the time requested to achieve the adult stage (Copepodite 5 to Copepodite 6) were evaluated, because the latter has been suggested as a suitable trait for detecting the effects of pollutants (Brown et al., 2002; Gutierrez et al., 2010). Differences between control and treatments in each one of the aforementioned endpoints were tested using one-way ANOVA, followed by a Tukey-Kramer multiple comparisons post Test (95% confidence level) (Sokal and Rohlf, 1969). Before each analysis, the normal distribution of data was verified with the Kolmogorov-Smirnov test. All statistical analyses were carried out using the package GraphPad InStat (InfoStat, 2004).

Results

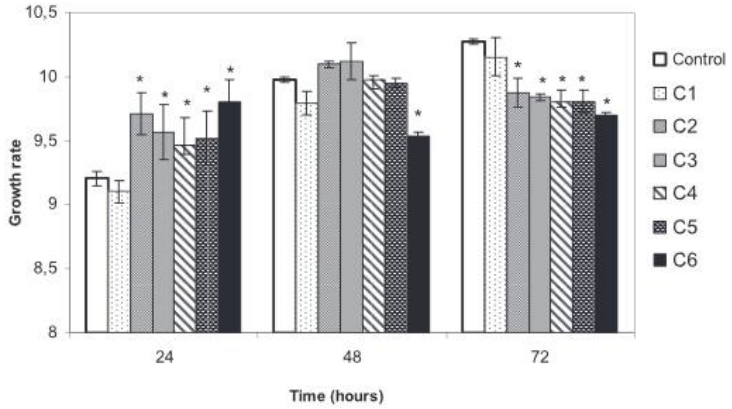
(N-(phosphonomethyl)glycine) Acute Toxicity.
The sensitivity of organisms varied among species. According to the acute toxicity test results, the order of sensitivity of the three species to GLY was: *S. vetulus* > *C. vulgaris*>. *N. conifer* (Table 1).

Table 1. Effective concentration (72-hour EC_{50} and 48-hour LC_{50}) of the glyphosate-based herbicide Eskoba to the alga *C. vulgaris*, the cladoceran *S. vetulus*, and the copepod *N. conifer*

Studied species	LC_{50} (mg/L)	EC_{50} (mg/L)	Exposure time (h)	Confidence interval (95%)
Simocephalus vetulus	21.5	–	48	13.9–30.8
Chlorella vulgaris	–	58.59	72	16.47–70.42
Notodiaptomus conifer	95.2	–	48	71.8–128.2

Toxicity of (N-(phosphonomethyl)glycine) to C. vulgaris
The increase of the herbicide concentration and the extension of the exposure time reduced the *C. vulgaris* growth rate (μ). After 24 hours of exposure, a slight tendency to stimulation of cell proliferation was produced in the higher GLY concentrations (ANOVA repeated measures, F = 3.94; $p < 0.05$). Conversely, no significant differences were found between the lower one and the control (Figure 1). After 48 hours of exposure, 16 mg/L of GLY inhibited the division of *C. vulgaris* cells (ANOVA repeated measures, F = 6.09; $p < 0.01$) (Figure 1). After 96 hours, this inhibition was very significant in the five higher concentrations (ANOVA repeated measures, $p < 0.01$) (Figure 1).

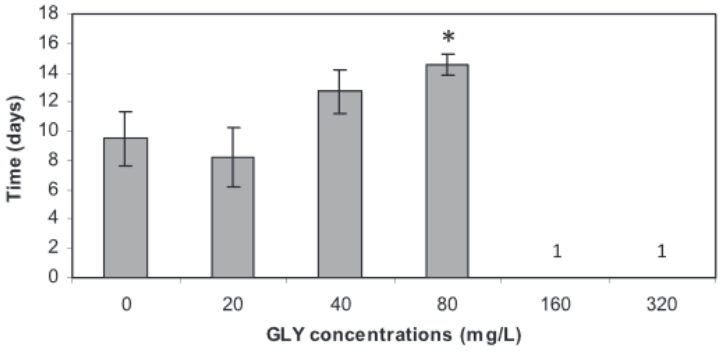
Figure 1. Growth rate (l) of *C. vulgaris* exposed to six concentrations of GLY and the control (without GLY): 0 mg/L(Control), 0.5 mg/L (C1), 1 mg/L (C2), 2 mg/L (C3), 4 mg/L (C4), 8 mg/L (C5), and 16 mg/L (C6) during 24, 48, and 72 hours. Error bars indicate the (6) standard deviation (three replicates per treatment). An asterisk (‘‘*’’) denotes significant differences of the control (one-way ANOVA, p < 0.05).



Recovery Experiments with Microcrustaceans

For *S. vetulus*, the mortality in the control was 10%; in other words, 27 organisms began the recovery test. On the other hand, after acute exposure to 3.2, 6.4, 12.8, 25.6 and 51.2 mg/L of GLY, the number of remaining survival organisms that entered the recovery test was 24, 21, 20, 17 and 9 respectively. At the end of the recovery tests without GLY, mortality was 100% in all the concentrations tested, with the exception of the lower concentration (3.2 mg/L), for which mortality was 86.7%. For organisms that have been exposed to 3.2 mg/L, their fecundity and age at first reproduction did not show significant differences with the control (ANOVA, $p < 0.05$). However, in GLY concentrations of 6.4 and 12.8 mg/L, the age at first reproduction was delayed 2 to 4 days (ANOVA, $p < 0.05$) (Table 2) and the average number of neonates/females was significantly reduced (ANOVA, $p < 0.05$). In the highest concentrations (25.6 and 51.2 mg/L of GLY), no reproduction was recorded (Figure 2). For copepods, the mortality in the control was 20%; in other words, 16 organisms began the recovery test. On the other hand, after acute exposure to 20, 40, 80, 160, and 320 mg/L of GLY, the number of remaining survival organisms that entered the recovery test was 20, 16, 12, 7, and 1, respectively. At the end of the recovery tests, the mortality for each one of the concentrations tested was 45, 12, 25, 57, and 0%, respectively. As can be seen, in the recovery tests, the mortality was strongly dependent on the number of organisms that could survive after the acute exposure tests. After being exposed to GLY concentrations of 160 and 320 mg/L, *N. conifer* could not reach the adult stage. In copepods exposed to 20 and 40 mg/L, this endpoint was not different from that of the control group (ANOVA, $p < 0.05$). Conversely, 80 mg/L of GLY significantly delayed the copepods' sexual maturity ANOVA, $p < 0.05$) (Figure 3).

Figure 3. Time (in days) at which the copepod *N. conifer* reaches the adult stage (from copepodite 5 to copepodite 6) after being exposed to five concentrations of GLY and the control (without GLY) for 48 hours. Vertical bars indicate the (6) standard deviation. An asterisk (''*'') denotes significant differences with the control (one-way ANOVA, $p < 0.05$). A''1''indicates that the organisms did not reach the adult stage.



Conclusion

The results of the present work suggest that the glyphosate formulation tested (48-hour LC50: 21.5 and 95.2 mg/L for *S. vetulus* and *N. conifer*, respectively; 72-hour EC_{50}: 58.59 mg/L for *C. vulgaris*) promotes harmful effects on native non-target species. To the authors' knowledge, this is the first work recording and comparing the changes in the life cycle of planktonic species that belongs to different trophic levels, caused by short-term exposure (48 hours) to a glyphosate formulation. This work also highlights the importance of assessing the effects of commercial formulations of GLY on aquatic species and the need to clearly indicate the additives of glyphosate-based herbicide commercial formulations. It was found in the literature that Roundup and the surfactant polyoxyethylene amine (POEA) were always more toxic than the active ingredient. Finally, this study demonstrates the importance of increasing information on the ranges of tolerance to GLY of organisms belonging to the planktonic community, and emphasizes the urgency of knowing its effects on wild species.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Overall the study was well described and conducted partially in accordance with OECD Guideline No. 201. The endpoints measured within the study included survival, age of reproduction, and fecundity. However, validity criteria on mortality was exceeded in the control organisms for several intervals. Analytical measurements were only performed on stock solution. Second part of the study was performed with native micro-crustacean species in Argentina. The test exposure history to the organisms was unknown as they were collected within the local environment. Finally, the glyphosate formulation Eskoba was tested, which is not the representative formulation for the glyphosate EU renewal. Due to the test materials not being the representative formulation, the study is not relevant for the glyphosate EU renewal.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The study is well described however analytical measurements were only performed on stock solution and not in tested concentrations. More importantly, Eskoba was used. In view of the formulation description, the use of the results to assess the toxicity of glyphosate as formulated in MON52276 is questionable. The study will be considered as less relevant but supplementary (formulation issue).

Acute effects of Eskoba® on the microalgae *Chlorella vulgaris*, the cladoceran *Simocephalus vetulus*, and the copepod *Notodiaptomus conifer* were investigated. The recovery ability of the surviving microcrustaceans was also assessed. Survival, age of first reproduction, and fecundity were used as endpoints for *S. vetulus*, while survival and time to reach the adult stage were used as endpoints for *N. conifer*.

S. vetulus: 48-hour EC50 = 21 mg/L
C. vulgaris: 72-hour EC50 = 58.59 mg/ L
N. *conifer*: 48-hour EC50 = 95 mg/L

Despite the growth of *C. vulgaris* stimulated after 24 hours of exposure to the commercial formulation of glyphosate Eskoba®, it was inhibited after 48 hours by all the concentrations tested.
In post-exposure experiments, microcrustaceans reduced their life expectancy, *S. vetulus* decreased its fertility, and *N. conifer* inhibited its sexual maturity.

RMS considers all these parameters relevant.

Therefore RMS considers that this study is less relevant but supplementary (due to the different formulation tested) and reliable with restrictions.

B.9.2.8. Further testing on aquatic organisms

B.9.2.8.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.2.4
Report author	Avigliano L. *et al*.
Report year	2014

Report title	Effects of glyphosate on growth rate, metabolic rate and energy reserves of early juvenile crayfish, *Cherax quadricarinatus* M.
Document Source	Bulletin of environmental contamination and toxicology (2014), Vol. 92, No. 6, pp. 631
Guidelines followed in study	Standard procedures recommended by the APHA et al. (2005) - 10600 Fishes
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Early juveniles of the crayfish *Cherax quadricarinatus* were exposed for 60 days to 10 and 40 mg/L of pure glyphosate (acid form) in freshwater. Mortality was 33 % at the highest concentration, while no differences in moulting were noted among treatments. After the first month of exposure, weight gain was significantly ($p < 0.05$) reduced in the 40 mg/L group. At the end of the assay, lipid levels in muscle, as well as protein level in both hepatopancreas and muscle were significantly ($p < 0.05$) reduced. These results suggest long-term utilization of both lipid and protein as main energetic reserves, likely in response to the chronic stress associated with herbicide exposure. Besides, the lower pyruvate kinase activity in muscle suggests a possible metabolic depression in this tissue. The hemolymphatic ASAT:ALAT ratio showed higher levels than the control at the highest glyphosate concentration, indicating possible damage to several tissues.

Materials and methods

Cherax quadricarinatus early juveniles (mean body weight = 1.28 ± 0.06 g, n = 36) were obtained from ovigerous females mated in the laboratory; females were purchased from a commercial hatchery (Pinzas Rojas S.R.L, Tucumán, Argentina). A chronic (60-days) bioassay was conducted in semi static conditions, according to standard procedures recommended by the American Public Health Association et al. (2005). Temperature was maintained at 26 ± 1°C throughout, while photoperiod was held at 14:10 (L:D). Dilution water used was prepared from tap water (hardness 80 mg/L as $CaCO_3$ equivalents, pH = 8.0 ± 0.5) purified through a series of three filters with replaceable cartridges (Hidroquil®) to retain sediment, organic matter (by activated charcoal), and cations (using a cationic resin). The water was dechlorinated by holding it for at least 48 h in a storage tank. Dissolved oxygen was always < 5 mg/L. Twelve juveniles were randomly assigned to each glyphosate concentration or dilution water control, each juvenile was placed in a 13-cm diameter glass jar, filled with 400 mL of dilution water control and provided with both a small PVC pipe and a tiny plastic net as refuges. Water of all recipients was completely changed three times a week, i.e., mostly every 48 h; 6 h before the change of water, all animals were fed food pellets (35 % protein, 11.12 % lipids, 20.84 % carbohydrates) prepared in the laboratory. Food pellets were provided to the crayfish in an amount equivalent to 5 % of body mass, supplemented with *Elodea* sp. fresh leaves, *ad libitum*.

Nominal concentrations used were 10 and 40 mg/L of pure glyphosate (acid form, 99.8 % purity, Sigma Co., St. Louis, Missouri). Stock solution was prepared weekly by dissolving the appropriate amount of glyphosate in distilled water. The pH of stock solution was always corrected by adding drops of a 10 N NaOH solution, to achieve the same pH value than the one mentioned for dilution control.

Nominal concentrations in the experimental recipients were validated after 48 h of aging, in order to estimate the possible loss of glyphosate between two successive water changes. To this purpose, duplicate samples from each glyphosate concentration was measured by means of ionic chromatography. Results of this validation are shown in Table 1.

During the assay, both mortality and molting were registered daily. The molted exoskeleton was kept for 48 h in each container to allow the animal to feed on it. Additionally, all animals were weighed every 2 weeks, in order to calculate the weight gain (WG) as WG = (FW-IW)/IW × 100, where IW is the initial weight and FW is the final weight of juveniles. At the end of the assay, oxygen consumption, hemolymphatic glucose level and energy reserves (glycogen, protein, and lipids) were determined in all surviving animals. Also, specific activity of both pyruvate kinase (PYK) and lactate dehydrogenase (LDH) were assessed in both muscle and hepatopancreas of all surviving crayfish. Finally, both ALAT and ASAT activities were determined in hemolymph extracted from all surviving crayfish with 30 min of incubation, measuring the absorbance of the pyruvate-DNPH complex at 490 nm.

To test significant differences between means, a one-way ANOVA followed by LSD multiple comparisons test (Sokal and Rohlf, 1981) was used. Data normality and homogeneity of variances were always confirmed. Fisher exact test was used to compare the proportion of dead or molted crayfish. A 5% confidence level was considered in all cases.

Results

The highest mortality value (33%) was seen in animals exposed to 40 mg/L of glyphosate, this value was significantly ($p < 0.05$) different from that of control; no significant ($p > 0.05$) differences were noted in the molting percentages between either glyphosate concentrations and the control (Table 1).

Table 1. Nominal versus measured concentrations of glyphosate in 48 h-aged solutions, as well as mortality and molting at the end of the 60-days exposure period.

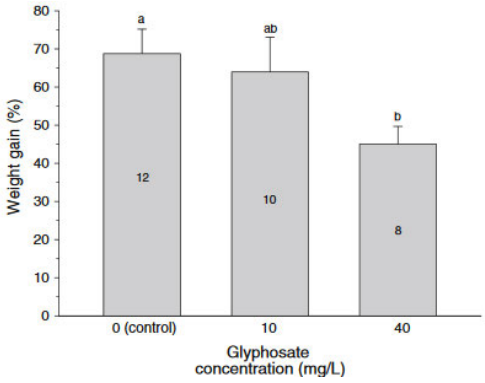
Nominal concentration (mg/L)	Measured concentration (48 h)	Mortality (%)	Molting (%)	
			1st molt	2nd molt
0 (control)	0	0	91.7	33.3
10	9.03 ± 0.03	16.7	75	16.7
40	40.13 ± 8.83	33.3*	75	50

Each measured concentrations represents the mean of two independent samples

* Significant ($p < 0.05$) differences with respect to control. Initial number of crayfish: 12 per treatment

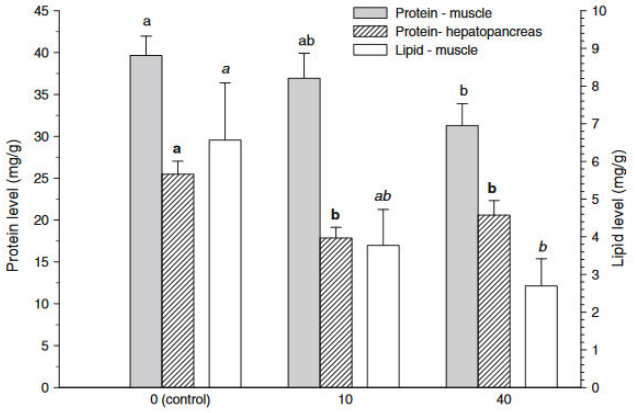
Figure 1 shows the WG values at the end of the assay, for both concentrations of glyphosate and the control. A clear and significant decrease in weight gain (35% lower than control) was seen after the first month of exposure to 40 mg/L of glyphosate.

Figure 1. Weight gain at the end of the experiment. In all cases, mean ± standard error is indicated. Different letters indicate significant (p < 0.05) differences. Number of animals (n) is indicated inside each bar



Concomitant with the observed decrease in weight gain, a significant ($p < 0.05$) decrease in total protein content (Fig. 2) was observed in both muscle, at 40 mg/L, and hepatopancreas, at both assayed concentrations. Besides, a significant ($p < 0.05$) decrease in total lipid content was clearly observed in muscle, at the highest glyphosate concentration, with respect to control (Fig. 2).

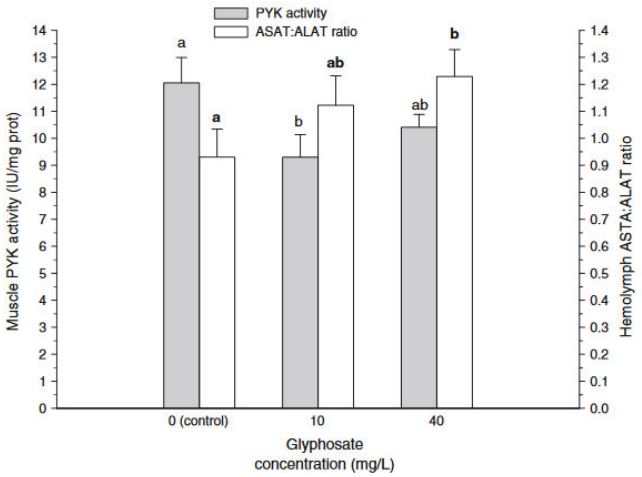
Figure 2. Total protein levels (mg/g) in abdominal muscle and hepatopancreas, and total lipids in muscle at the end of the experiment. In all cases, mean ± standard error is indicated. Different letters (same style) indicate significant (p < 0.05) differences. n = same as Fig.1).



No significant ($p > 0.05$) differences were noted among treatments, concerning, glycaemia or glycogen content.

At the 10 mg/L exposure, muscle pyruvate kinase activities were significantly lower ($p < 0.05$) relative to the control group (Fig. 3), while no differences ($p > 0.05$) were seen in the hepatopancreas. Concerning lactate dehydrogenase, no significant ($p > 0.05$) differences were seen in any case, suggesting that the anaerobic pathway in this tissue remains inactive. Taken together, these results were indicative of a reduced total metabolic intensity of cells. Given that no differences were seen in the oxygen consumption of the whole organism, such reduction cannot be a priori extended to tissues other than muscle.

Figure 3. PYK activity (IU/mg protein) in abdominal muscle, as well as hemolymphatic ASAT:ALAT ratio, at the end of the experiment, In all cases, mean ± standard error is indicated. Different letters(same style) indicate significant ($p < 0.05$) differences. n = same as Fig.1



ASAT:ALAT ratios measured in hemolymph at 40 mg/ L exposure concentrations were significantly ($p < 0.05$) higher relative to controls (Fig. 3). The significantly higher ASAT:ALAT ratio found at the highest glyphosate concentration suggests that many organs are being affected by glyphosate in their cellular integrity.

Conclusion

Glyphosate was able to reduce growth rates and protein and lipid reserves in chronically exposed early juvenile crayfish.
Although most of the effects (decrease in weight gain, protein reserves in both hepatopancreas and muscle and lipid reserves from muscle, as well as the increase in ASAT:ALAT hemolymphatic level) were seen at a glyphosate concentration of 40 mg/L, some effects (decrease in protein reserves in hepatopancreas and an apparent metabolic depression in muscle) were observed at 10 mg/L, a concentration near reported environmental concentrations.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Enzyme, cellular and molecular level endpoints are not relevant for the EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal. In addition, mortality and survival data were not discussed.

Assessment and conclusion by RMS:

The main objective of the study was to assess the effects of sublethal concentrations of glyphosate on early juvenile of the crayfish *Cherax quadricarinatus*, in terms of growth rate, metabolic rate and energy reserves levels, to determine how glyphosate affects the activity level of key metabolic enzymes, such as pyruvate kinase and to determine the levels of both alanine and aspartate aminotransferase activities (ALAT and ASAT respectively) as indicative of tissue damage. All the experiments were done with pure glyphosate (acid form). The study is considered relevant to assess effects of glyphosate on non target organisms.

The highest mortality value (33 %) was seen in animals exposed to 40 mg/L of glyphosate, this value was significantly different from control; A significant decrease in weight gain (35 % lower than control) was seen after the first month of exposure to 40 mg/L of glyphosate. Significant decrease in total protein content in both muscle, at 40 mg/L, and hepatopancreas, at both assayed concentrations. Besides, a significant decrease in total lipid content was observed in muscle. At the 10 mg/L exposure, muscle pyruvate kinase activities were significantly lower (while no differences were seen in the hepatopancreas.
Both lipids and proteins are closely involved with the energy available for crustacean growth.
So RMS considers that these parameters are relevant even if not used in the current scheme of risk assessment.
This study states that glyphosate is able to reduce growth rates and protein and lipid reserves in chronically exposed (60 days, semi-static, concentrations were maintained) early juvenile crayfish at concentrations of 40 mg/L. Some effects (decrease in protein reserves in hepatopancreas and an apparent metabolic depression in muscle) were observed at 10 mg/L. Such concentrations are far above the concentrations expected fin environmental concentrations for the uses intended.
The exposure levels are deemed unrealistic by RMS. The reliability of the study for regulatory risk assessment purpose is low.
Overall, RMS agrees to classify the study as relevant and reliable with restrictions after detailed assessment of full-text article.

Data point:	CA 9
Report author	Avigliano L. *et al.*
Report year	2018
Report title	Effects of Glyphosate on Somatic and Ovarian Growth in the Estuarine Crab *Neohelice granulata*, During the Pre-Reproductive Period
Document No	Water, air, and soil pollution (2018), Vol. 229, No. 2, pp. 44
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: **as provided in the AIR5 dossier (KCA 9) as proposed by applicant:** **See RMS analysis in RMS comment box**	Not relevant by full text

Full summary of the study according to OECD format

Adult females of the estuarine crab *Neohelice granulata* were exposed during the 3-month pre-reproductive period (winter) to the herbicide glyphosate, the most used pesticide in Argentina, at three different concentrations (0.02, 0.2, and 1 mg/L, as active ingredient). At the end of the *in vivo* assay, the body weight gain and the ovarian growth were estimated, the last one in terms of the gonadosomatic index (GSI), the relative proportion of the different kind of oocytes, and their relative size. A decrease in the body weight gain was observed by effect of pure glyphosate, at all concentrations assayed. Although no differences in either the GSI or vitellogenic protein content of the ovary were noted between any glyphosate concentration and control, a higher proportion of reabsorbed vitellogenic oocytes was observed in the ovaries of crabs exposed to glyphosate at 1 mg/L, together with an increased area of previtellogenic oocytes. These effects were confirmed in vitro, at a glyphosate concentration of 0.2 mg/L. In fact, a higher area of previtellogenic oocytes was seen when glyphosate was added to the culture medium containing ovarian tissue, but a significant higher incidence of reabsorbed vitellogenic oocytes was seen only when eyestalk tissue was also added to the vials, suggesting that the secretion of some neurohormone involved in reabsorption is enhanced. The obtained results indicate that glyphosate is able to harm, in the studied species, both somatic and the ovarian growth.

Materials and methods

In vivo Experiment

Adult females of *N. granulata* were randomly collected in June (body weight = 10.35 ± 0.12 g, N = 75), at the southern edge of Samborombón Bay, a non-polluted area at the mouth of the Río de la Plata estuary, Argentina. All bioassays were conducted in semi-static conditions. Briefly, water from all test recipients was completely renewed twice a week, always maintaining the water quality parameters and laboratory conditions mentioned below.

Concentrated stock solutions of pure glyphosate (as acid, 99.8% purity; Sigma, Missouri) were prepared weekly by dissolving the appropriate amount of the chemicals in distilled water, to avoid precipitation. Small aliquots from these stock solutions were then added to the test recipients, previously filled with the dilution saline water detailed below. Nominal concen trations of 0.02, 0.2, and 1 mg/L were assayed. In order to validate nominal concentrations, water samples (15 mL) were taken at 0 and 72 h, i.e., the period for water replacement in all test containers. After derivatization at pH=9 with 9-fluorenylmethylchloroformate (FMOC-CL), glyphosate concentrations were measured HPLC/MS, detector Agilent, model VL. A X-SELECT C18 chromatographic column. A mixture of MeOH/NH_4 5 mmM/9 mM was chosen as mobile phase, with a flow rate of 0.5 mL/min.

Fifteen females were assigned to each treatment, i.e., control (with no toxic added) or treated with glyphosate at 0.02, 0.2, or 1 mg/L. For all experiments, each female was isolated in a glass container filled with 400 mL of saline water prepared by diluting artificial seawater salts (Tetra Marine Salt Pro, US) in dechlorinated tap water (hardness: 80 mg/L as equivalents of $CaCO_3$; final salinity, 12 g/L, pH=8.0 ± 0.5) provided with constant aeration. The solution of each container was completely replaced twice a week. A temperature of 23 ± 1 °C and a photoperiod of 14:10 (L/D) were maintained in the aquarium. All animals were fed twice a week with pellets prepared in the laboratory.

Females remained exposed to all glyphosate concentrations or control for 90 days, i.e., throughout the entire pre-reproductive period (June 24 to September 23). In order to evaluate the ovarian growth prior to spawning, ovigerous females were not considered for further analysis. Females that molted during the experiment were also excluded taking into account that the energy invested for molting could represent a disadvantage for the ovarian growth. Therefore, at the end of the assays, non-ovigerous and non-molted females were weighed to determine body weight (BW), which were later compared to the initial body weight, in order to estimate weight gain (WG) as ((final BW-initial BW)/initial BW) × 100. Animals were finally sacrificed after anesthetizing them in ice water, and both ovaries and hepatopancreas were dissected and weighed in order to determine the gonadosomatic (GSI) and hepatosomatic (HSI) index as GSI or HSI = (GW or HW)/final BW) × 100, where GW and HW are the gonad and hepatopancreas wet weight, respectively. In addition, both the GSI and HSI were measured in an initial sample of crabs (10.78 ± 0.05 g body weight, N = 15).

Dissected ovaries were fixed in Bouin solution for 4 h at room temperature, dehydrated in alcohol series, and finally embedded in paraplast. Then, 5-μm sections were prepared and stained with hematoxylin and eosin. Previtellogenic, intermediate, and vitellogenic oocytes were characterized according to their

size and degree of basophilia. To assess the proportions of the different kind of oocytes, a grid of 100 points was used, counting the number of points included in each oocyte type, at a 40× magnification; at least three ovarian sections from each animal, taken from the thickest part of the ovary, were examined by this procedure. Considering the ellipsoidal shape of oocytes, both the major (M) and minor (m) diameter of every oocyte showing its nucleus were measured in the same histological sections above mentioned, in order to estimate the oocyte area. For this, a micrometric ocular lens, calibrated against a Leitz Wetzlar plate with 1/100 mm spacing, was used. As in previous studies (Rodríguez et al. 1994), the oocyte area was calculated as $(\pi/4) \times M \times m$.

An ELISA assay was employed to determine the total content of vitellogenic proteins (Vg: vitellogenin and vitellins). A primary antibody against native Vg was obtained by inoculating rabbits with purified Vg Anti-IgG from rabbit, conjugated with peroxidase (BIOARS Lab.), was used as the secondary antibody. Purified Vg in a 1/500 dilution was used to prepare the standard curve (0–210 ng). Fifty microliters of either the standard or sample was placed, in triplicate, in a 96-well plate (Nunc-Immunoplate Polisorp). Samples were previously diluted in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH=9.6). Both primary and secondary antibodies were diluted (1/500) in PBS + 0.05% Tween + 6% powder milk. Absorbance was measured in all wells at 415 nm, by using an ELISA-plates reader (BIO-Rad Lab., Model 680); 2-20-Azino-di-3-ethylbenzthiazoline sulfonic acid was used as chromogen.

In Vitro Experiments

During June, stock female crabs were used for the in vitro experiments. Ovarian explants (approximately 1 × 0.5 cm each) were incubated for 24 h in 2-mL vials, inside culture chambers held at 27 °C, constant darkness and with CO_2 at 5%. Medium 199 was prepared using powdered medium with L-glutamine and Earle's salts (Sigma Chemical Co.), dissolved in crustacean saline (Cooke et al. 1977), and modified to compensate for the salts already present in this culture medium. As in previous studies (Sarojini et al. 1997; Rodríguez et al. 2000), 6 mg of penicillin-G per 100 mL of medium was added, and the pH was adjusted to 7.4 with 0.5 N NaOH.

Ten females were assigned to each experimental series, each female providing a similar piece of ovary to every treatment (blocking design). One series consisted of only ovary, either with the addition of pure glyphosate at 0.2 mg/L or with no glyphosate added (control); a second series involved the same treatments but every eyestalk of each sacrificed female was also added, to either control or glyphosate-treated vial. To this, eyestalks were cut off at their articulation with cephalothorax and below the ommatidia; after gently dissecting the cuticle, the soft tissue was washed and assigned to vials. At the end of the 24-h incubation period, all ovarian pieces were fixed and processed for histological analysis, following the same methodology described for the in vivo assays.

Statistical Analysis

A one-way ANOVA followed by LSD pairwise comparisons (Sokal and Rohlf 1981) was used for testing differences between experimental groups concerning both the proportion of each oocyte type and the ovarian vitellogenin content. Logarithmic or angular transformation of data was eventually used when homogeneity of variances was not confirmed in raw data. Proportion of survival, molted or ovigerous females were compared between experimental groups by means of the Fisher exact test (Sokal and Rohlf 1981).

Results

Measured glyphosate concentrations were close to the nominal ones; no significant degradation seems to occur at 72 h of aging, i.e., before replacing all test solutions.

Table 1 Nominal and measured concentrations of glyphosate, used in the in vivo assay

Nominal concentration (mg/L)	Measured concentration (mg/L)		
	0 h	72 h	Overall mean
0.02	0.0186 0.0225	0.0299 0.0269	0.0245 ± 0.0025
0.2	0.1786 0.1795	0.2108 0.2604	0.2073 ± 0.0192
1	0.8502 0.9009	1.6673 1.6449	1.2658 ± 0.2256

Mortality was low in almost all treatments, although it rose up to 33.33% at the lowest glyphosate concentration ($p < 0.05$, compared to control). At the highest concentration assayed, the percentages of both molting and ovigerous females reached 20 and 26.67%, respectively, which were not different ($p > 0.05$) from control values. All the females that became ovigerous during the experiment lost the spawned eggs during the first days of incubation; these eggs were not fertilized, according to the verification made under stereoscopic lupe (50×), indicating that used females had no spermatophores stored in their spermathecae.

Table 1. Molting, spawning and mortality of females at the end of the *in vivo* assay.

Glyphosate concentration (mg/L)	Ni	% mortality	% molting	% ovígerous	Nf
0 (control)	15	0.00	6.67	13.33	12
0.02	15	33.33 *	13.33	6.67	7
0.2	15	6.67	13.33	20.00	9
1	15	6.67	20.00	26.67	7

Ni initial number of females, *Nf* final number, considering only the non-molted, non-ovigerous surviving (NM-NO) females. Mortality percentage is always referred to NM-NO females

Compared to control, WG was significantly ($p < 0.05$) lower at all glyphosate concentrations tested. Although no significant differences ($p > 0.05$) were observed in either the gonsadosomatic index or hepatosomatic index, a tendency toward a reduction of GSI was observed as glyphosate concentration increased. No significant differences ($p > 0.05$) were noted in both the vitellogenic protein content of the ovary (overall mean: 1969.11 ± 202.15 μg/g of ovary) and its circulating levels (overall mean: 91.93 ± 7.93 μg/ mL of hemolymph).

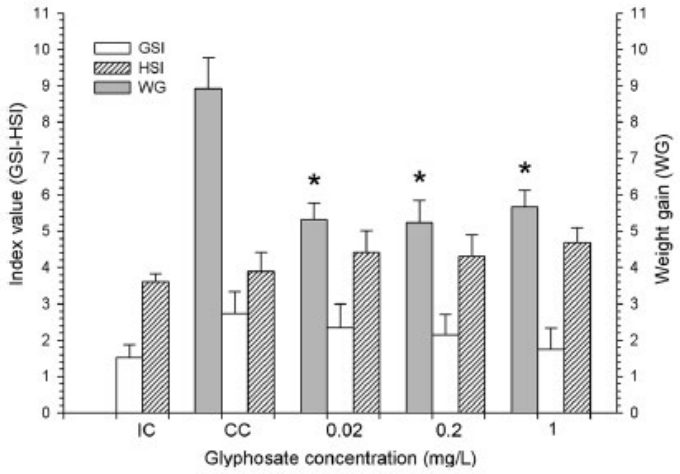


Fig. 1 Weight gain (WG), gonadosomatic (GSI), and hepatoxomatic (HIS) index of females at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. IC = initial control; CC = concurrent control. Asterisks indicate significant differences ($p < 0.05$) with respect to control (CC)

Concerning the proportion of oocyte types in the ovary observed at the end of the *in vivo* experiment, a significantly ($p < 0.05$) higher proportion of reabsorbed vitellogenic oocytes was observed at the highest glyphosate concentration, together with a lower proportion of normal vitellogenic oocytes.

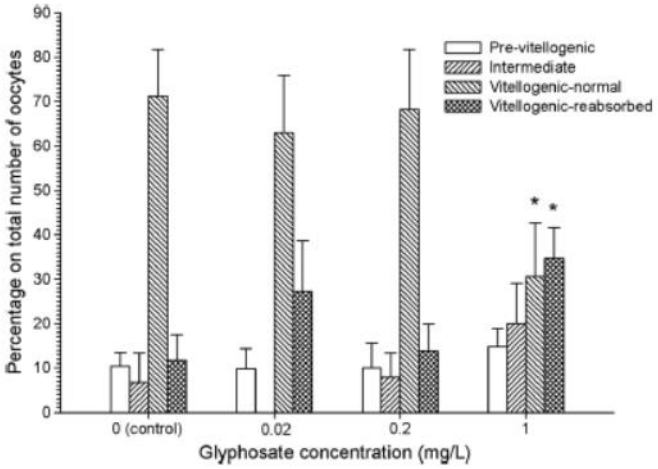


Fig. 2 Proportion of oocyte type in the ovary (mean ± SE) of females, at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. Asterisks indicate significant differences ($p < 0.05$) with respect to control

On the other hand, the area of normal previtellogenic oocytes was significantly ($p < 0.05$) augmented by effect of pure glyphosate at 1 mg/L. The results of the *in vitro* experiments confirmed that previtellogenic oocytes reached an area significantly ($p < 0.05$) bigger than that of control, when glyphosate was added to the culture medium at a concentration of 0.2 mg/L. When eyestalk tissue was added, though, no significant differences ($p > 0.05$) were noted in oocyte's area, but a significant ($p < 0.05$) percentage of reabsorbed vitellogenic oocytes was noted.

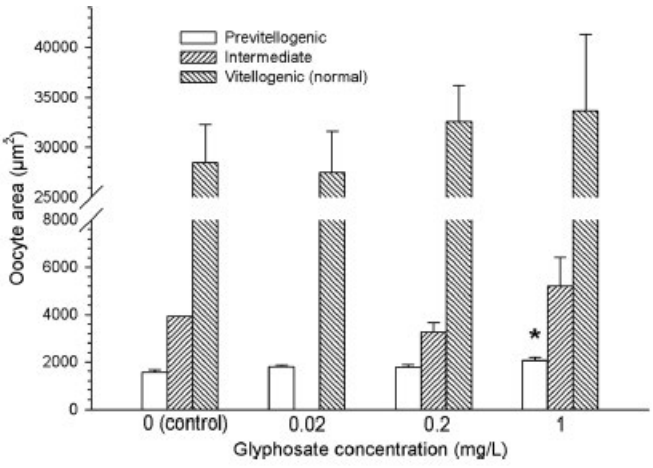


Fig. 3 Relative area of oocytes in the ovary (mean ± SE) of females, at the end of the in vivo experiment. Number of females (Nf) is indicated in Table 2. Asterisks indicate significant differences ($p < 0.05$) with respect to control

Conclusion

Glyphosate was able to produce, at relatively low concentrations, several harmful effects on adult female crabs during the pre-reproductive period. Firstly, the weight gain of adults was reduced *in vivo* by effect of pure glyphosate, at a concentration as low as 0.02 mg/L. In the ovary, the exposure to glyphosate caused, both *in vivo* (at 1 mg/L) and *in vitro* (at 0.2 mg/L), a significant reabsorption of vitellogenic oocytes, together with an increase in the area of previtellogenic oocytes. The *in vitro* experiments suggest that the active ingredient glyphosate could be causing some imbalances in the endocrine control of ovarian growth.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: It is difficult to relate the findings of the study to an EU level ecotoxicology risk assessment as they are based on GSI and HIS values and the different types of oocyte found in the ovaries between exposure groups.
Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU).

Assessment and conclusion by RMS:

Adult females of the estuarine crab Neohelice granulata were exposed during the 3-month pre-reproductive period (winter) to the herbicide glyphosate, at three different concentrations (0.02, 0.2, and 1 mg/L, as active ingredient).
RMS considers these concentrations environmentally realistic.

A decrease in the body weight gain on adult female crab was observed by effect of pure glyphosate, at all concentrations assayed (NOEC < 0.02 mg/L). It is likely due to treatment but does not appear concentration related.
Higher proportion of reabsorbed vitellogenic oocytes was observed in the ovaries of crabs exposed to glyphosate at 1 mg/L, together with an increased area of previtellogenic oocytes. These effects were confirmed in vitro, at a glyphosate concentration of 0.2 mg/L. This study suggests that glyphosate could be causing some imbalances in the endocrine control of ovarian growth.
Based on the above, the study is considered relevant for the assessment of potential for endocrine disruption.

Concerning reliability of the results, several shortcomings are noted:
The study did not follow standardized guideline but seems well conducted. Only graphs were presented.
Concentrations were analytically verified.
The concentrations of 0.02, 0.2, and 1 mg/L were assayed as these had been shown to be sublethal (Avigliano et al. 2014). RMS notes that results on mortality are not consistent (33.33, 6.67 and 6.67% mortality of females respectively), so the MTC cannot be determined.
Concerning the potential impact of using wild-caught organisms, particularly regarding potential effects on the endocrine system from prior exposure to other substances, it is mentioned in the report that the specimens were randomly collected at the southern edge of Samborombón Bay, a "nonpolluted" area at the mouth of the Rio de la Plata estuary, Argentina. The contamination level cannot be verified by RMS, it is only stated that very scarce information about glyphosate environmental levels has been published, with reported values between 0.1 mg/L and 0.7 mg/L in water and between 0.5 mg/kg and 5 mg/kg in sediments. However, the report also states (in the introduction) that "*heavy charge of herbicides and other pesticides is carried by several rivers and channels that cross extensive agricultural areas and finally reach the Samborombón Bay (Comisión Administrativa del Río de La Plata 1990)*". RMS then cannot discard the presence of other toxicants in this estuary.

Overall, it is not possible to relate the observed effects to an endocrine mode of action (MTC not defined).

The study is relevant but considered reliable with restrictions (for effects on bodyweight gain, not reliable for endocrine properties).

Data point:	CA 8.2.4
Report author	Demetrio P. M. *et al*.
Report year	2012
Report title	Effects of pesticide formulations and active ingredients on the coelenterate *Hydra attenuata* (Pallas, 1766).
Document Source	Bulletin of environmental contamination and toxicology (2012), Vol. 88, No. 1, pp. 15-19
Guidelines followed in study	None
Deviations from current test guideline	Not applicable.

GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Lethal effects of the active ingredient and a glyphosate formulation Roundup® Max commonly used in soybean cultivation were assessed with the *Hydra attenuata* toxicity test. Glyphosate formulation exhibited higher toxicity at low concentrations ($LC_{1–10}$) respect to active ingredient, reversing this behavior at higher concentrations ($LC_{50–90}$). Comparing *H. attenuata* sensitivity with existent toxicity data for aquatic organisms indicates that this species is highly sensitive to glyphosate.

Materials and methods

Hydra attenuata was obtained from the Watertox Bioassays Program of the International Development Research Center of Canada. Hydra was maintained and grown in glass bowls containing 0.5 L of Hydra medium at 20 ± 1°C with a 16 h light and 8 h dark photoperiod.

Acute toxicity tests with individual toxicants (active ingredient and formulation) were performed with *H. attenuata* in quadruplicate using a minimum of five concentrations of each toxicant in culture water as dilution media. Tests were done in multiplates of 3 mL wells, exposing three organisms per well during 96 h with no renewal of test solutions. Temperature and light conditions were 21 ± 2°C and 16:8 L:D, respectively. Assessed endpoints were lethality (irreversible malformation like the tulip stage and the disintegration of organisms). The acceptability criterion of the tests was normal morphology appearance in all organisms of negative controls. Routine sensitivity controls were performed using Cr(VI) as the reference toxicant prepared from the salt $K_2Cr_2O_7$ (Sigma-Aldrich Analytical Reagent). Glyphosate (Technical Grade) was obtained from Gleba S.A. (Buenos Aires, Argentina), and the herbicide Roundup® Max (74.4% glyphosate) was obtained from Monsanto S.A. (St. Louis, Missouri, USA).

Stocks solutions (100 mg/L) were done in distilled water and used immediately to prepare test dilutions in testing water. Concentrations in stocks were measured by chromatographic methods (HPLC/MS). Verification of the maximum and minimum concentrations in testing dilutions (n = 4 per treatment) was also performed at the beginning and end of exposure. For each set of samples a procedural blank and a matrix sample spiked with standards were used to determine the accuracy. The whole treatment recoveries were over 75% for all the tested compounds.

Calculation of $LC_{1;5;10;15;50;85}$ values was done using Probit model (Finney 1971) with a specific software (Probit USEPA version 1.5). Slope and elevation comparisons were performed to assess differences in toxicity between the active compound and the formulation.

Results and discussion

Measured concentrations in stock solutions of formulation and a.i were, respectively 98.3 ± 1.0 and 97.8 ± 1.1 mg/L of glyphosate. Tests were carried within the following measured concentration ranges: 13.7 – 36.1 and 15.8 – 22.2 for formulation or active ingredient, respectively. A maximum of 20% decay in concentration during 96 h testing period was detected.

Results of toxicity tests with *H. attenuata* for the formulation and active ingredient ($LC_{1;5;10;15;50;85}$ values) are shown in Table 1. Regression and correlation analyses for the log-probit model of

formulation versus active ingredient are shown in Table 2. Glyphosate and Roundup® Max showed significant differences among the slopes. When the formulation is compared with the a.i., the ratio between observed effects for both forms in different points of the curves are not constant.

Table 1. Acute toxicity data for active ingredient and formulation. Results correspond to 96 h exposure and are expressed in mg/L of a.i. The 95% CI are given between parentheses.

LC_x	Glyphosate formulation	Glyphosate a.i.
1	10.7 (7.1–13.3)	14.8 (12.6–15.9)
5	13.3 (9.7–15.6)	15.7 (13.9–16.6)
10	14.8 (11.4–17.0)	16.2 (14.7–17.0)
15	15.9 (12.8–18.1)	16.6 (15.2–17.3)
50	21.8 (19.5–24.2)	18.2 (17.4–18.9)
85	29.9 (26.7–36.3)	20.0 (19.5–22.2)

Table 2. Regression and correlation parameters of concentration response plots. *form* = formulation, *a.i.* = active ingredient.

Regression parameters	Glyphosate	
	Form	a.i.
b	7.58	25.86
a	−5.16	−27.59
n	7	5
r	0.89	0.95
r^2	0.80	0.91

Glyphosate at low percentages of effect exhibits no differences (compared across the limits of confidence) between formulation and a.i.; but at high percentages of mortality, (> 50%) differences are observed (Table 1). For glyphosate, statistical significant differences (a = 0.05) are observed for the slope, that is higher for the a.i. (Table 3).

Table 3. Regression and correlation analysis for comparisons. *Dif* = differences, *a.i.* = active ingredient, *R* = reject, *A* = accept.

Formulated versus a.i.	Glyphosate
Dif. among slopes	2.79
Critical value	2.31
Null hypothesis $b_1 = b_2$	R
Dif. among elevations	0.65
Critical value	2.26
Null hypothesis $a_1 = a_2$	A

The sensitivity of *H. attenuata* to glyphosate was compared with toxicity intervals of LC_{50} values for a set of selected freshwater species obtained from US EPA (2007). These data were organized by species giving 36 values for glyphosate. Toxicity database intervals for the 10th and 90th percentiles were 6,200-673,430 µg/L for glyphosate; indicating that sensitivity of *H. attenuata* to glyphosate is very high to the herbicide (below the 20th percentile).

The toxicity of glyphosate has been reported for a few non-photosynthetic and photosynthetic organisms such as bacteria, protozoans, insects, fish, amphibians, algae and vascular plants. Reports indicate a toxicity pattern from higher toxicity in low complexity organisms (bacteria through hydra) and photosynthetic ones, and low toxicity in fish and amphibians. This fact contributes to the hypothesis that the effect of the herbicide active ingredient on the metabolic pathway of hydra would be different from that corresponding to other, more complex organisms. The shikimic acid metabolic pathway is assumed to be absent in animals. However, it has been recently reported that the genes associated with the enzymes required in the shikimic pathway could also be present in marine cnidarians, suggesting an explanation to the observed toxicity of glyphosate to hydra.

Conclusion

Higher and significant effects on *H. attenuata* were detected for the glyphosate formulation compared to the active ingredient at lower concentrations, with a reversal of the behavior at higher concentrations. The general pattern of the biological effects on the coelenterate is different from that observed for other animal species belonging to higher organization levels, with *H. attenuata* being very sensitive to glyphosate.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Endpoints for *Hydra attenuata* are not a data requirement for the glyphosate EU renewal / data requirements under 1107/2009. The tested formulation is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
This study aims to assess the lethal effects of glyphosate and glyphosate formulation Roundup® Max on the *Hydra attenuate* (96 hours).
Analytical verifications have been made.
The endpoints were reported to be :
96h-LC50 glyphosate a.i =18.2 mg a.i/L
96h-LC50 RoundupMax® =21.8 mg a.i/L (considered less relevant but supplementary by RMS due to the different formulation tested)
The study seems well conducted (despite the absence of specific guideline) however there are no details of biological observations reported in the paper. Thus, the observed mortality and the LC50 calculation cannot be confirmed by RMS. The results suggest that RoundupMax® is as toxic as glyphosate.
This study is reliable with restrictions.

Data point:	CA 8.2.8
Report author	Mottier A. et al.
Report year	2013
Report title	Effects of glyphosate-based herbicides on embryo-larval development and metamorphosis in the Pacific oyster, *Crassostrea gigas*.

Document Source	Aquatic toxicology (2013), Vol. 128-129, pp. 67
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

The present study aimed to assess the toxicity of glyphosate, its by-product, aminomethylphosphonic acid (AMPA) and two commercial formulations, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}), containing glyphosate as the active ingredient, on the early life stages of the Pacific oyster, *Crassostrea gigas*. The embryotoxicity of these chemicals were quantified by considering both the rates of abnormalities and the arrested development or types of abnormalities in D-shaped larvae after 48 h exposure. The success of metamorphosis was examined in pediveliger larvae exposed for 24 h. Experiments involving both endpoints included range finding experiments for herbicide concentrations ranging from 0.1 to 100,000 µg/L. This range was then narrowed down in order to determine precise EC_{50} values. Actual concentrations of the herbicide were determined at the beginning and after 48 h (embryotoxicity) and 24 h (metamorphosis) to evaluate the potential temporal variation in the concentrations. During embryo-larval development, no mortalities were recorded at any of the concentrations of glyphosate and AMPA, whereas no embryos or D-shaped larvae could be observed after exposure to 10,000 µg/L of R_{EX} or R_{AT}. Compared with the controls, no effects on embryo-larval development were recorded between 0.1 and 1000 µg/L, regardless of the chemical tested. Above a threshold, which varied according to the chemical used, the gradient of herbicide concentrations correlated with a gradient of severity of abnormality ranging from normal larvae to arrested development (an "old embryo" stage). The EC_{50} values were 28,315 and 40,617 µg/L for glyphosate and its metabolite, respectively, but much lowered values of 1133 and 1675 µg/L for R_{EX} and R_{AT}, respectively. Metamorphosis tests also revealed a significant difference between molecules, as the EC_{50} values exceeded 100,000 µg/L for glyphosate and AMPA but were as low as 6366 and 6060 µg/L for the commercial formulations, which appeared relatively more toxic. Overall, the embryo-larval development of *C. gigas* was more sensitive to glyphosate-based herbicides compared to various endpoints studied in regulatory model organisms, and embryos and D-shaped larvae were more sensitive compared to pediveliger larvae.

Materials and Methods

Chemical compounds

In this study, the effects of two commercial Roundup® herbicides were assessed, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}), which contain 7.2 and 4.4 g/L of glyphosate, respectively. These formulations contain adjuvants polyethoxylated tallow amines (POEA). In the present study, glyphosate (97% purity) and AMPA (97.5% purity) were obtained from Dr. Ehrenstorfer GmbH® (Augsburg, Germany). For both endpoints, the nominal concentrations corresponding to 0.1, 1, 100 and 10,000 µg/L of the chemicals (i.e. glyphosate and AMPA) were verified (in duplicate) by ultraperformance liquid chromatography (UPLC) and fluorometric detection (in accordance with NF ISO 21458) using UPLC Acquity with FLR detector (Waters) and a column Acquity BEH C18-2.1 mm × 150 mm, 1.7 µm. Moreover, the analyses were performed at the beginning and at the end of the exposures to verify the variation in the tested concentrations during the period of the experiment. These analyses were performed once without embryos or larvae to avoid the interaction between the physico-

chemical and biological processes. Finally, the concentrations of glyphosate and AMPA in both commercial formulations (R_{EX} and R_{AT}) were determined (5 replicates) to verify the values indicated on the labels.

Embryotoxicity bioassay and experimental design

Embryo-larval toxicity tests were performed on oyster embryos exposed to herbicides using the standardized AFNOR procedure published in 2009. Four experiments were conducted, and for each experiment, herbicide concentrations were tested in triplicate. For the first two experiments, herbicide concentrations ranged from 0.1 to 100,000 µg/L with a factor of 10× between each two consecutive concentrations (7 concentrations in total + control). Then, embryos were exposed to additional concentrations of herbicides corresponding to narrower ranges to more precisely determine the EC_{50} values. For glyphosate and AMPA, the narrowed range was from 10,000 to 30,000 µg/L, and 9 and 11 different concentrations (+ control) were tested, respectively. For each commercial formulation, the narrowed range was between 1000 and 2000 µg/L, and a total of 18 different concentrations (+ control) were tested. Apart from the herbicide exposures and the control, $CuSO_4 \cdot 5H_2O$ was used as a positive control, with concentrations ranging from 20 to 100 µg/L (5 concentrations).

Conditioned oysters were purchased from the Guernsey Sea Farm Ltd. hatchery (Guernsey, UK). Male and female gametes were obtained by thermal stimulation of 3 pairs of genitors (successive baths at 16 °C or 28 °C). After spawning, gametes from the different genitors were observed under a light microscope to select genitors with the best cell qualities: highly concentrated and motile spermatozoa and even, pear-shaped oocytes. The spermatozoa and oocytes of the selected parents were passed through, respectively, 40 and 100 µm screens to remove debris. Female and male gametes (1:6) were then mixed and gently agitated. The occurrence of fertilisation was verified with light microscope observations, and 20-min post-fertilisation, the embryos were distributed into glass pillboxes containing 25 mL of natural sterilised seawater. Embryos were exposed at a density of 60,000/L (corresponding to 1500 embryos per pillbox) without feeding, aeration and light. After 48 h at 22 ± 1 °C, embryos or D-shaped larvae were fixed using 0.5 mL of an 8% formalin solution.

An average of 100 larvae was counted per replicate using an inverted binocular microscope at 400× magnification. Observations allowed the calculation of rates of abnormality and the discrimination among types of abnormalities; 5 categories could be distinguished: shell and/or hinge abnormality, mantle abnormality (hypertrophies), shell and/or hinge + mantle abnormality, late arrested development and early arrested development (when cells could be distinguished and counted).

Metamorphosis bioassay and experimental design

The aim of this endpoint was to assess the metamorphosis rate of pediveliger larvae (ready for metamorphosis) exposed to herbicides. Experiments were performed 4 times, and each herbicide concentration was tested at least in triplicate. For the four experiments using glyphosate and AMPA, herbicide concentrations tested ranged from 0.1 to 100,000 µg/L (broad range). For the Roundup® formulations, the two first experiments were conducted on the broad range of concentrations; then, pediveliger larvae were exposed to a narrow range of concentrations between 1000 and 10,000 µg/L (19 concentrations + control). Twenty one-day-old pediveliger larvae were purchased from the SATMAR (Societe ATlantique de MARiculture) hatchery (Barfleur, France).

Larvae were exposed in multiwell plates in a final volume of 1.5 mL of natural sterilized seawater. Larval density was set between 50 and 80 larvae per well. Experiments were conducted for 24 h at 22 °C without feeding, aeration and light. After 24 h, exposed larvae were observed using an inverted binocular microscope at 100x magnification to count dead larvae that showed no movement and/or tissue degradations. Following this first count, larvae were fixed using an 8% formalin solution. The metamorphosis rate was evaluated by counting metamorphosed versus non-metamorphosed larvae. A larva was considered metamorphosed when it presented an obvious loss of its velum, new shell growth and well-developed gills. Aside from metamorphosis processes, mortality was also examined, considering the latter "more serious" than a lack of metamorphosis.

Statistical analysis

The comparison of herbicide concentrations at the beginning of the experiments and after 24 h (metamorphosis test) or 48 h (embryotoxicity) exposure was performed using Mann-Whitney tests. Non-linear regressions (using the Hill equation) on data obtained from the two endpoints allowed to calculate

various EC_X (effective concentration for an effect on X% of the individuals tested) values for each contaminant. These regressions were conducted using the Excel® macro REGTOX (Vindimian, 2012). Data related to the effects of glyphosate and AMPA on metamorphosis were statistically tested by one-way ANOVAs, as they met the assumptions of parametric tests (normal distribution and homogeneity of variances). On the other hand, the data on the impact of commercial formulations on metamorphosis and all of the data concerning embryotoxicity (even transformed) did not meet these assumptions; thus, Kruskal-Wallis tests were employed to make conclusions about the significance of the differences between both the controls and the various herbicide concentrations and the herbicide concentrations themselves. When ANOVA or Kruskal-Wallis tests revealed significant differences, multiple comparison tests (Student-Newman-Keuls; SNK or SNK modified for Kruskal-Wallis test) were then undertaken to distinguish among different groups. All of the analyses were conducted using STATISTICA 8.0 software.

Results

Analyses of the tested molecules

For the glyphosate and AMPA exposures, the measured concentrations in general were slightly higher than the nominal concentrations (Table 1); these differences were greater for the lowest concentrations (1 and above all 0.10 µg/L) and reached a maximum of 40% (0.14 µg/L instead of 0.10 µg/L) for the embryotoxicity test at T0h. However, the measured concentrations were globally close to the nominal concentrations. The glyphosate concentrations of Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}) averaged 9.52 µg/L (±1.23) instead of 7.20 g/L, and 6.27 g/L (±1.63) instead of 4.40 g/L, respectively. It thus appeared that the commercial formulations were overdosed by +32.22% for R_{EX} and +42.50% for R_{AT}. For the exposures of embryos and larvae to Roundup®, the various solutions were prepared based on the advertised concentrations (i.e., 7.2 and 4.4 g/L); therefore, the true concentrations exceeded the nominal ones for both endpoints (Table 1). The maximal difference attained was 50% for the embryotoxicity test with R_{EX} at T0h and T48h (0.15 µg/L instead of 0.10 µg/L), but generally, the excess did not reach the proportion of 32.22% for R_{EX} and 42.50% for R_{AT}, especially for the highest concentration tested (10,000 µg/L). When the values recorded at the beginning of the experiments were compared with those measured after 24 or 48 h of exposure, no significant differences were calculated (Mann-Whitney tests, $p > 0.05$), and it could be concluded that the organisms were exposed to constant concentrations during the two types of experiments.

Table 1. Results (mean values in µg/L ± SEM) of the herbicide analyses performed for both endpoints at the beginning of the experiment and after 24 h or 48 h of exposure to glyphosate, aminomethylphosphonic acid (AMPA) and two commercial formulations: Roundup Express® and Roundup Allées et Terrasses®.

	Nominal concentrations	Embryotoxicity		Metamorphosis rate	
		T0h	T48h	T0h	T24h
Glyphosate ($\mu g L^{-1}$)	0.1	0.12 ± 0.01	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.01
	1	1.20 ± 0.07	1.20 ± 0.08	1.09 ± 0.01	1.15 ± 0.05
	100	114.1 ± 3.1	114.5 ± 3.9	109.7 ± 7.1	113.0 ± 1.4
	10,000	10,025 ± 113	9833 ± 244	10,407 ± 166	11,055 ± 155
AMPA ($\mu g L^{-1}$)	0.1	0.14 ± 0.00	0.14 ± 0.03	0.10 ± 0.00	0.14 ± 0.01
	1	1.09 ± 0.05	1.07 ± 0.01	1.04 ± 0.02	1.08 ± 0.00
	100	108.7 ± 2.8	103.4 ± 0.04	102.8 ± 0.1	104.1 ± 0.6
	10,000	10,552 ± 187	9957 ± 67	9805 ± 7	10,392 ± 39
R_{EX} ($\mu g L^{-1}$)	0.1	0.15 ± 0.02	0.15 ± 0.01	0.11 ± 0.01	0.12 ± 0.02
	1	1.21 ± 0.01	1.29 ± 0.00	1.07 ± 0.02	1.00 ± 0.00
	100	108.7 ± 2.6	122.6 ± 15.9	119.6 ± 16.5	110.9 ± 0.2
	10,000	10,625 ± 403	10,745 ± 49	10,455 ± 14	10,757 ± 272
R_{AT} ($\mu g L^{-1}$)	0.1	0.13 ± 0.00	0.14 ± 0.00	0.12 ± 0.02	0.11 ± 0.01
	1	1.25 ± 0.00	1.32 ± 0.01	1.25 ± 0.03	1.25 ± 0.03
	100	136.6 ± 4.8	135.6 ± 0.6	138.1 ± 12.7	116.3 ± 3.0
	10,000	10,652 ± 286	10,662 ± 11	12,140 ± 827	11,845 ± 537

Effects of glyphosate-based herbicides on embryo-larval development

For each experiment, the results of the embryo-larval bioassay revealed very high levels of fecundation, as almost no oocytes were recorded and the observed organisms were embryos and normal or abnormal D-larvae. Moreover, all of the embryotoxicity tests presented could be validated because they respected the two validation conditions required by the standardised procedure: in controls, the rate of normal larvae must reach at least 80%, and the EC_{50} Cu^{2+} must fall between 6 and 16 µg/L. Indeed, these parameters ranged, respectively, from 82.94% (SEM:±2.51%) to 92.08% (SEM:±1.48%), and from 8.47 to 12.43 µg/L.

Exposures to glyphosate (and AMPA) and to commercial formulations gave different results in terms of organism survival and embryo-larval development. No mortalities were observed at any of the concentrations of glyphosate and AMPA tested, even at 100,000 µg/L; however, at this highest concentration, only embryos and abnormal D-shaped larvae were present. In contrast, at 10,000 µg/L of R_{EX} and R_{AT}, no embryos or D-larvae were observed, and mortality rates of 100% were thus recorded.

In comparison to controls, no effects on embryo-larval development were recorded between 0.1 and 1000 µg/L regardless of the chemical studied. A drastic impact of herbicide exposure was observed between 1000 µg/L and 10,000 µg/L for both Roundup® formulations, whereas this hazardous effect occurred between 10,000 µg/L and 100,000 µg/L for glyphosate and AMPA. From 20,000 µg/L of glyphosate or AMPA, the rates of normal D-shaped larvae decreased, but compared to the lower concentrations, the differences were not significant (Kruskal-Wallis and SNK tests, $p > 0.05$) due to a high degree of heterogeneity. Compared to glyphosate, AMPA showed a higher EC_{50} value: 40,617 µg/L (versus 28,315 µg/L) (Table 2). Moreover, at the highest concentration (100,000 µg/L), glyphosate appeared to be slightly more toxic than AMPA because the net percentages of normal development (NPN_e) were 0.52% (SEM: ±0.45%) and 20.45% (SEM: ±6.88%), respectively. For the exposures to commercial formulations, no normal larvae were observed at the tested concentrations of 4000, 6000 and 8000 µg/L. This conclusion led to narrow the range of tested concentrations of R_{EX} and R_{AT} again, to between 1000 and 2000 µg/L. From the exposure to 1000 µg/L R_{EX}, NPN_e progressively decreased up to the concentration of 1500 µg/L, which did not allow normal larval development in *C. gigas*. For R_{AT} exposure, the NPN_e profile differed because no significant effects were observed up to 1600 µg/L, and a sharp decrease was then noted. Considering both the EC_{10} and the EC_{50} values (Table 2), the tested commercial formulations were more toxic than the active matter and its metabolite, and among the Roundup® formulations, R_{EX} was the most toxic with an EC_{50} of 1133 µg/L (versus 1672 µg/L for R_{AT}).

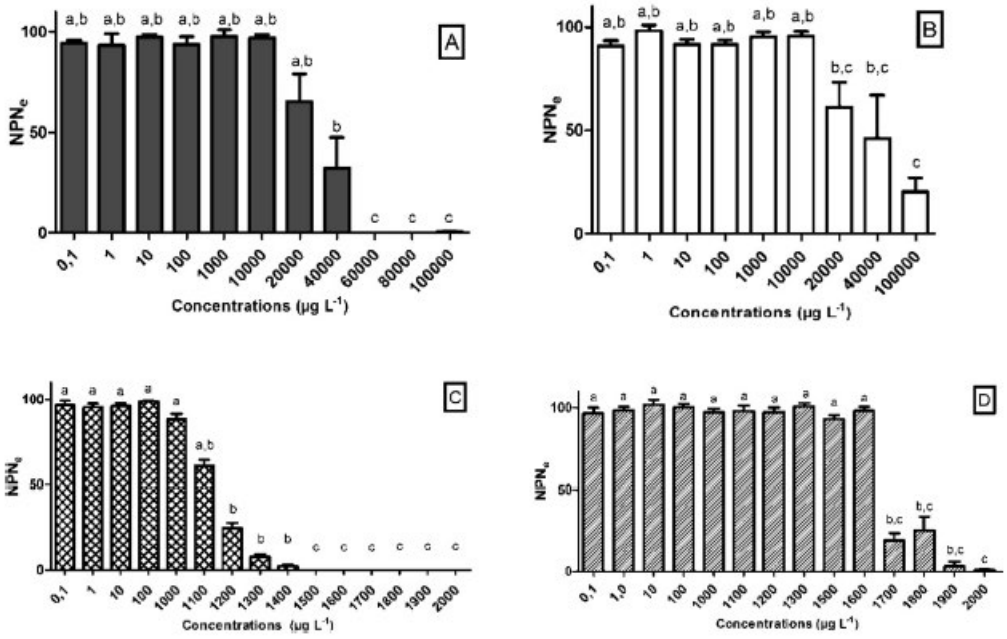


Fig. 3. Net percentages of normal development (NPN_e) (±SEM) in *C. gigas* embryo-larvae observed after 48 h of exposure to herbicides at concentrations ranging from 0.10 to 100,000 µg L^{-1} for glyphosate (A) and AMPA (B) or 2000 µg L^{-1} for Roundup Express® (R_{EX}) (C) and Roundup Allées et Terrasses® (R_{AT}) (D). Since no normal larvae were observed at the tested concentrations of 4000, 6000 and 8000 µg L^{-1} for R_{EX} and R_{AT}, they are therefore not represented. The concentrations that do not share a letter are significantly different; by convention, the controls belong to group a. For R_{AT}, NPN_e data lacking at 1400 µg L^{-1} due to a technical problem.

Table 2. Ecotoxicological parameters calculated for (1) the embryotoxicity tests (rates abnormalities in D-shaped larvae) and (2) the rates of pediveliger larvae mortality and metamorphosis after 48 h exposures to 4 herbicide substances: glyphosate (GLY), AMPA, Roundup Express® (R_{EX}) and Roundup Allées et Terrasses® (R_{AT}). ECX = effective concentration (in µg/L) which induces an effect on X% of the population (10 or 50%).

Endpoints	Parameters	GLY	AMPA	R_{EX}	R_{AT}
Abnormality rates in D-shaped larvae	EC_{10}	13,457	10,299	1006	1628
	EC_{50}	28,315	40,617	1133	1672
Mortality rates of pediveliger larvae	EC_{10}	>100,000	>100,000	6601	4991
	EC_{50}	>100,000	>100,000	8502	7934
Metamorphosis rates of pediveliger larvae	EC_{10}	>100,000	>100,000	5215	4150
	EC_{50}	>100,000	>100,000	6366	6060

Table 3. Ecotoxicological parameters corrected by considering the measured concentrations of herbicides (instead of the nominal concentrations; for comparison, see Table 2 for the embryotoxicity tests and the rates of pediveliger larvae metamorphosis). GLY = glyphosate; AMPA = glyphosate metabolite; REX = Roundup Express® and RAT = Roundup Allées et Terrasses®. ECX = effective concentration (in µg /L) that induces an effect on X% of the population (10 or 50%).

Endpoints	Parameters	GLY	AMPA	R_{EX}	R_{AT}
Abnormality rates in D-shaped larvae	EC_{10}	13,347	11,032	1037	1951
	EC_{50}	27,175	46,105	1168	2001
Metamorphosis rates of pediveliger larvae	EC_{10}	>100,000	>100,000	5778	5244
	EC_{50}	>100,000	>100,000	6940	7550

When abnormal embryo-larval development was observed, different types of abnormalities could be distinguished. In the controls and the range of herbicide concentrations no effects were recorded regardless of the chemical used (i.e., 0.1 to 1000 µg/L). The most frequent abnormalities (~4.3–6.3%) were arrested development at the "old embryo" stage (EMB) and abnormalities affecting the shell and/or hinge (SHEL). The percentages of abnormalities affecting the mantle (MANT) or both the shell and the mantle (MASH) significantly increased from approximately 1000 µg/L for the commercial formulations and 10,000 µg/L for glyphosate and AMPA. Exposures to R_{EX} could be distinguished by the frequency of these abnormalities. At the highest concentrations, frequent arrest of development at the "old embryo" stage was observed. The level of 50% "old embryos" was not attained for AMPA and occurred at ~40,000 µg/L for glyphosate, ~1850 µg/L for R_{AT} and ~1550 µg/L for R_{EX}.

Effects of glyphosate-based herbicides on larval metamorphosis

Aside from metamorphosis rates, mortality rates were also considered because mortality processes are "more serious" than a lack of metamorphosis. For the four metamorphosis tests performed, the mortality rate of the controls was 1.73% (SEM: ± 0.47%). For the exposures to glyphosate and AMPA, mortality rates were low whatever the concentration tested; they ranged, respectively, from 1.68% (SEM: ± 0.71%; 10 µg/L) to 7.49% (SEM: ±2.76%; 100,000 µg/L) and from 1.22% (SEM: ±0.61%; 10 µg/L) to 2.84% (SEM: ±1.03%; 1000 µg/L). In both cases, the EC_{50} value was higher than 100,000 µg/L (Table 2). By contrast, the tests applied to the commercial formulations revealed far higher mortality rates; EC_{50} values were as low as 8502 and 7934 µg/L for R_{EX} and R_{AT}, respectively.

For the 4 experiments, the metamorphosis rate of the controls reached 78.03% (SEM: ±1.50%). By comparison with mortality rates, the metamorphosis rates of pediveliger larvae showed similar differences between herbicide substances. Indeed, glyphosate and AMPA appeared far less toxic than the two commercial formulations, and for the former substances, the wide range tested (up to 100,000 µg/L) did not allow to determine the EC_{50} values (Table 2). Although no significant differences were found among the different concentrations of AMPA (ANOVA, $p = 0.08$), the highest concentration of glyphosate induced a slight but significant decrease in the metamorphosis rate to 80.15% (SEM:±5.50%) (ANOVA, $p < 0.001$; SNK, $p < 0.01$). The exposures to R_{AT} showed a significant decrease of the metamorphosis rate with increases in the R_{AT} concentration above 4000 µg/L (Kruskal-Wallis, $p < 0.001$). Two rather low concentrations (1 and 10 µg/L) led to slight decreases in the metamorphosis rate, which did not differ significantly from the rate recorded at 4000-6800 µg/L (group b); however, for these two low concentrations, the standard errors were particularly high. The profile of metamorphosis

rates recorded for R_{EX} exposures was similar to that of R_{AT} exposures, and from 5000 µg/L, the metamorphosis rate significantly decreased with an increasing concentration of R_{EX} (Kruskal-Wallis, $p < 0.001$). Finally, for the two types of Roundup®, the EC_{50} showed similar values: 6366 µg/L and 6060 µg/L for R_{EX} and R_{AT}, respectively (Table 2).

Conclusion

During embryo-larval development, no mortalities were recorded at any of the concentrations of glyphosate and AMPA, whereas no embryos or D-shaped larvae could be observed after exposure to 10,000 µg/L of R_{EX} or R_{AT}. Compared with the controls, no effects on embryo-larval development were recorded between 0.1 and 1000 µg/L, regardless of the chemical tested. Above a threshold, which varied according to the chemical used, the gradient of herbicide concentrations correlated with a gradient of severity of abnormality ranging from normal larvae to arrested development (an "old embryo" stage). The EC_{50} values were 28,315 and 40,617 µg/L for glyphosate and its metabolite, respectively, but much lowered values of 1133 and 1675 µg/L for R_{EX} and R_{AT}, respectively. Metamorphosis tests also revealed a significant difference between molecules, as the EC_{50} values exceeded 100,000 µg/L for glyphosate and AMPA but were as low as 6366 and 6060 µg/L for the commercial formulations, which appeared relatively more toxic. Overall, the embryo-larval development of *C. gigas* was more sensitive to glyphosate-based herbicides compared to various endpoints studied in regulatory model organisms, and embryos and D-shaped larvae were more sensitive compared to pediveliger larvae.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study was not conducted to GLP and/or according to a recognized test guideline and there are no validity criteria presented. The authors state that the EC_{50} values computed for the embryotoxicity tests with glyphosate and AMPA were lower than the values reported for regulatory model organisms. The embryotoxicity test appeared more sensitive but also a more difficult to assess compared to the metamorphosis assay. Given the limitations cited, the study is considered unreliable.

Further points of clarification:
The formulations used in this study contain POEA surfactant which is not permitted in formulated herbicidal products in the EU. Therefore this study is not considered relevant to the glyphosate EU renewal and the findings in this paper should be treated with high level of caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The study aimed to assess the toxicity of glyphosate, its by-product, aminomethylphosphonic acid (AMPA) and two commercial formulations, Roundup Express® (REX) and Roundup Allées et Terrasses® (RAT), containing glyphosate as the active ingredient, on the early life stages of the Pacific oyster, Crassostrea gigas. Embryotoxicity bioassay.
This is marine species (marine species are also relevant for risk assessment).

The EC50 values were 27.1 and 46.1 mg/Lfor glyphosate and AMPA, respectively for the parameter development (Abnormality rates in D-shaped larvae, measured concentrations)
The EC10 values were 13.457 and 10.299 mg/Lfor glyphosate and AMPA,
Glyphosate and AMPA LC50 >100mg/L (based on measured concentrations)

Results of the two commercial formulations, Roundup Express® (REX) and Roundup Allées et Terrasses® (RAT), are not considered by RMS as the authors indicated that these formulations contain POEA and are not relevant.

This study was not conducted according to a recognized test guideline and there are no validity criteria presented. Analytical verifications have been performed. The study appears to be well-performed and reported.
RMS considers the study relevant and reliable for active substance data.

Data point:	CA 9
Report author	Baker L. F. *et al.*
Report year	2014
Report title	The direct and indirect effects of a glyphosate-based herbicide and nutrients on Chironomidae (Diptera) emerging from small wetlands.
Document No	Environmental toxicology and chemistry (2014), Vol. 33, No. 9, pp. 2076-85
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

Using a replicated, split-wetland experiment, the authors examined the effects of 2 nominal concentrations (2.88 mg a.e./L and 0.21 mg acid equivalents/L) of the glyphosate herbicide Roundup WeatherMax, alone or in combination with nutrient additions, on the emergence of Chironomidae (Diptera) before and after herbicide-induced damage to macrophytes. There were no direct effects of treatment on the structure of the Chironomidae community or on the overall emergence rates. However, after macrophyte cover declined as a result of herbicide application, there were statistically significant

increases in emergence in all but the highest herbicide treatment, which had also received no nutrients. There was a negative relationship between chironomid abundance and macrophyte cover on the treated sides of wetlands. Fertilizer application did not appear to compound the effects of the herbicide treatments. Although direct toxicity of Roundup WeatherMax was not apparent, the authors observed longer-term impacts, suggesting that the indirect effects of this herbicide deserve more consideration when assessing the ecological risk of using herbicides in proximity to wetlands.

Materials and methods

Test substances: Roundup WeatherMax

Test design / Exposure concentrations:

The 24 wetlands used in the present study were located in the Long-Term Experimental Wetlands area, a 4-km2 area approximately 60 km northwest of Saint John, New Brunswick, Canada. This area had never been treated with any chemical pesticides prior to the present study.
24 wetlands were divided into two halves using a high density polyethylene (HDPE) geomembrane (Poly-Flex Geomembrane Lining Systems) sheeting. 1 m height with sealed pockets filled with crushed gravel along the bottom to anchor into the sediments, stretching the entire length of the wetland and extending beyond the high water mark. One-half of the wetlands (n = 12) received a predicted maximum environmental concentration of 2.88 mg a.e./L. This nominal concentration was chosen based on the predicted environmental concentration of the active chemical in a 15-cm water depth if a wetland had been directly oversprayed with no intercepting vegetation at the maximum label rate for use on perennial weeds (4.32 kg a.e./ha). The remaining 12 wetlands received an environmentally realistic concentration (0.21 mg a.e./L) of the herbicide (low). Two separate herbicide treatments were conducted over 2 consecutive days each (12 wetlands each day) on 15 and 16 May 2009, and 9 and 10 June 2009. The number and timing of herbicide treatments were consistent with common agricultural practices in the region. All control sides of wetlands were simultaneously sprayed with uncontaminated wetland water, using a separate sprayer, every time applications were made to the treated sides of wetlands.
One-half of each glyphosate treatment group was also handsprayed with nutrients commonly used in inorganic agricultural fertilizers (technical grades of ammonium nitrate and phosphoric acid; purchased from Fisher Scientific). Wetlands were generally classified as oligotrophic/mesotrophic. The full experimental design consisted of 6 H wetland halves (high concentration), 6 HN wetland halves (high concentration + nutrients), 6 L wetland halves (low concentration), and 6 LN wetland halves (low concentration + nutrients), each with a paired untreated half (48 experimental units in total), except in August, when only the wetlands containing water could be sampled.

Test species*:* Naturally occurring Chironomidae in wetland systems.

Chemical analysis: Water samples were collected from all experimental units every 2 week through the course of the summer and frozen in clean HDPE bottles for nutrient analysis (n = 1/wetland half/date). Ammonium ($NH4^+$-N) concentrations were measured by the Agriculture and Food Laboratory at the University of Guelph, Guelph, Ontario, Canada, following the US Environmental Protection Agency method, using a modified Berthelot reaction, on a SEAL AQ2 automated discrete nutrient analyzer (colorimeter; SEAL Analytical). Total phosphorus concentrations were measured by the Research and Productivity Council, Fredericton, New Brunswick, Canada. Method detection limits were 0.05 mg/L ($NH4^+$-N) and 2 µg/L (total phosphorus). Glyphosate concentrations were measured in water samples collected from both sides of each wetland on days 1, 3, and 7 after the first round of treatment and on days 0, 3, and 7 after the second round of treatment. Samples of the water column from 5 sites in each wetland half were composited in ethylene oxide-sterilized 50 mL polycarbonate centrifuge tubes and stored frozen until analysis (n = 1/wetland half/date). Glyphosate residues were quantified using a gas chromatograph (GC; Hewlett Packard HP 5890 Series II) with a nitrogen–phosphorus detector by the Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada. Known amounts of glufosinate ammonium were used as an internal standard, and concentrations of glyphosate were calculated in comparison to this standard. The quality of the method was checked by running natural field water samples spiked with known amounts of glyphosate and blank matrix samples. Percent recovery of spiked samples was within acceptable limits (87.6%, n = 18); the detection limit was 5 µg/L, and the limit of quantitation was 17 µg/L. All values (n = 21 of 72) below the detection limit were replaced with the value of the detection limit/2, which produces no bias in a small data set when approximately 25% of the values fall below the detection limit. The half-life of glyphosate in each treated wetland was calculated using the nominal concentration as the initial concentration (N_0), and the measured concentration (N_t) 3 d (t) after addition.

Wetland plant surveys: The percent cover of both emergent and submerged macrophyte species were estimated visually from multiple locations around the wetland. Data were standardized using a comparison chart for visual estimation of percent cover, and plants were identified to species. Herbicide treatments were conducted early in the growing season, before sufficiently mature plant material was available to estimate percent cover. For this reason, plant surveys were conducted in late May, between the initial and final herbicide treatments, and twice more after the final herbicide treatment, in late June and in August 2009. The May plant survey was not done at the same time as the chironomid sampling, and therefore, these data were not compared statistically.

Chironomidae emergence collection:

Emerging adult chironomids were collected from wetlands with preservative-free emergence traps. Each emergence trap consisted of a cone constructed of 200-µm mesh size, sheer, white, polyester fabric. A bamboo stake was installed in the sediment of the wetland, and the trap was placed over the stake so that the open end of the trap was resting against the water surface, enclosing an area of 706 cm^2. Traps were deployed for 4 d to 7 d and frozen for at least 24 h to kill chironomids. Whole chironomids were removed, preserved in 70%

ethanol, enumerated, and identified to the subfamily/tribe level using a dissecting microscope. On each side of each wetland, 3 to 5 traps were deployed.
Sets of traps were deployed in each experimental unit (each wetland half) once before herbicide treatment, in spring 2009 (6–12 May 2009), and 3 times after both herbicide treatments (25 June–4 July 2009, 29 July–14 Aug 2009, and 13–23 April 2010). Spring arrived earlier in 2010 than in 2009; thus, the timing of temporally appropriate replicates was based on the comparative phenology of wood frogs (*Lithobates sylvaticus*), an important indicator species in the wetlands. The abundance of emerging chironomids from each experimental unit from each deployment was standardized to the average number per square meter per day.

Environmental conditions: Not stated

Statistical analysis: In the present study, experimental units were wetland halves (n = 48). To maintain the power of the paired experimental design, the abundance of emerging chironomids was expressed as the difference between treated and control sides, relative to the value on the control side for each wetland. Similarity of Chironomidae assemblage composition between sides of each wetland was expressed using a semiquantitative measure of community composition, the additive inverse of the Bray-Curtis dissimilarity index, which examines the differences in the relative abundances of mutually present taxa and ignores mutual absences. The formula was

$$\text{Bray–Curtis} = 100 \times (1 - [2C_{ij}/\{S_i + S_j\}])$$

where C_{ij} is the lesser sum of the abundance of species shared between 2 sites, S_i is the total abundance of species at site i, and S_j is the total abundance of species at site j. The average similarity, within sampling periods, between the control sides of independent wetlands was used as a baseline comparison at the landscape level. Two-tailed, 1-sample t tests were used to assess significant differences in relative chironomid abundance between treated and control sides of wetlands. Only declines were expected in similarity of the chironomid assemblage and in macrophyte cover as a result of chemical treatments; therefore, 1-tailed paired t tests were used to check for significant declines in chironomid assemblage similarity between the pretreatment and posttreatment sampling periods and to check for significant reduction in percent macrophyte cover on treated sides of ponds relative to control sides. Two-tailed, 2-sample t tests were used to examine differences in chironomid assemblage similarity between the herbicide-only and herbicide plus nutrients treatments in the June and August sampling periods.
Linear regressions were used to determine the relationship between the abundance of emerging chironomids and the percent cover of macrophytes for all treated sides of experimental wetlands compared with the untreated sides. The critical effect size $R^2 = 0.40$ was used, which is the equivalent of a critical effect size of 1.64 SD.

Results

Nutrient and glyphosate concentrations
Measured nutrient concentrations in wetland water were lower than anticipated during the treatment period (Fig. 1A). On average, NH_4 in treated wetland halves was below detection limits prior to

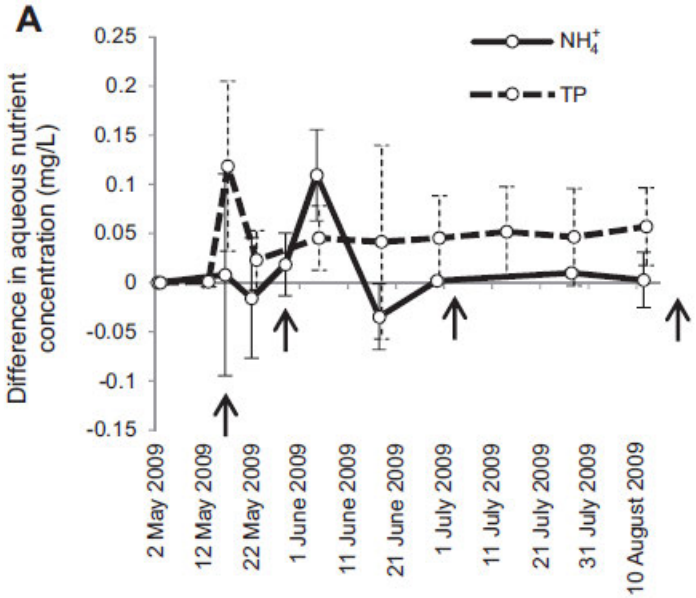
treatment. Nutrient additions led to transient increases in NH_4^+, and nutrient-treated sides of wetlands had, on average, 0.006 ±0.015 mg/L higher NH_4^+ than the paired control sides. There was negligible difference in total phosphorus between wetland sides prior to application of nutrients. During the treatment period, the concentration of total phosphorus increased on the treated sides of wetlands by an average 0.023 ± 0.014 mg/L when compared with paired controls.

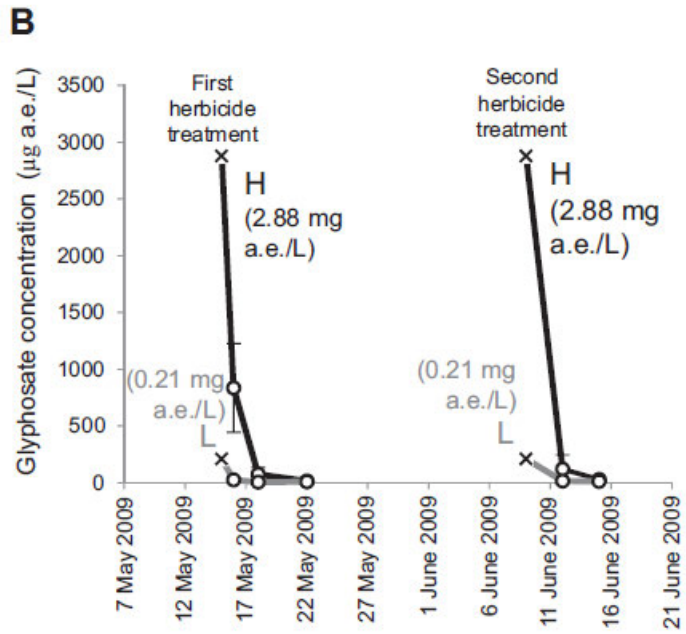
The concentration of glyphosate in the water column of the treated sides of experimental wetlands declined rapidly and was mostly nondetectable 7 d after either herbicide addition. The half-life of glyphosate in wetland water was approximately 0.56 d and 0.69 d following the first and second treatment applications, respectively. One day after the first treatment, there was significantly more glyphosate in all of the H-treated sides than in the L-treated sides of wetlands (all Hs 835.57 ±390.68 µg a.e./L; all Ls 26.69 ±12.62 µg a.e./L; p= 0.001, n= 12, 12, α= 0.068, 2-sample, 1-tailed, independent *t* test). At 3 d after the first treatment, only the H-treated wetlands had a detectable concentration of glyphosate of 75.88 ± 56.59 µg a.e./L, and at 7 d after treatment, neither treatment category had an average concentration of glyphosate above the limit of detection. After the second spray, only H-treated wetlands had an average concentration of glyphosate above the limit of detection at both 3 d and 7 d posttreatment (3 d 120.56 ±123.01 µg a.e./L, 7 d 28.66 ±19.55 µg a.e./L).

Figure 1.

(A) The difference (treated vs control) in aqueous ammonia (NH4+-N) and total phosphorus (TP) concentrations between treated and control sides of all nutrient-treated (HN [black] and LN [gray] treatments) split-wetlands (n=12;± 90% confidence interval). Arrows indicate the dates where nutrients were added to the treated sides of wetlands.

(B) Aqueous glyphosate concentrations on the treated sides of wetlands (±90% confidence interval; n=12 for each treatment). Initial nominal concentrations are indicated by an x on each curve; analytically measured concentrations are indicated with open circles. a.e.= acid equivalents; HN= higher glyphosate concentration plus nutrients; LN= lower glyphosate concentration plus nutrients.





Effects on macrophyte cover

In late May 2009, mean differences in the percent cover of macrophytes between the treated and control sides of wetlands were small (no greater than 5% for any treatment), and only the HN treatment sides differed significantly from 0 (H: p = 0.371, n = 6, α = 0.097; HN: p = 0.068, n = 6, α = 0.097; L: p = 0.207, n = 6, α = 0.097; LN: p = 0.208, n = 4, α = 0.160; all by paired, 1-tailed t tests). The minor differences between the treated and control sides in late May 2009 (7–13 d after herbicide treatment) were consistent with the expected 3-wk delay in plant damage after glyphosate application. Following the second herbicide application, macrophyte cover declined in every treatment category (H: p=0.009, n=5, α=0.124; HN: P=0.002, n=5, α =0.124; L: p=0.118, n=5, α =0.124; LN: p=0.002, n=6, α =0.097; all by paired, 1-tailed t tests). Similarly, in August 2009, macrophyte cover was significantly lower on the treated than the paired control sides of the wetlands that did not go dry (H: p=0.078, n=3, α =0.212; HN: p=0.036, n=4, α =0.160: L: p=0.055, n=3, α =0.212; LN: p=0.118, n=3, α =0.212; all by paired, 1-tailed t tests).

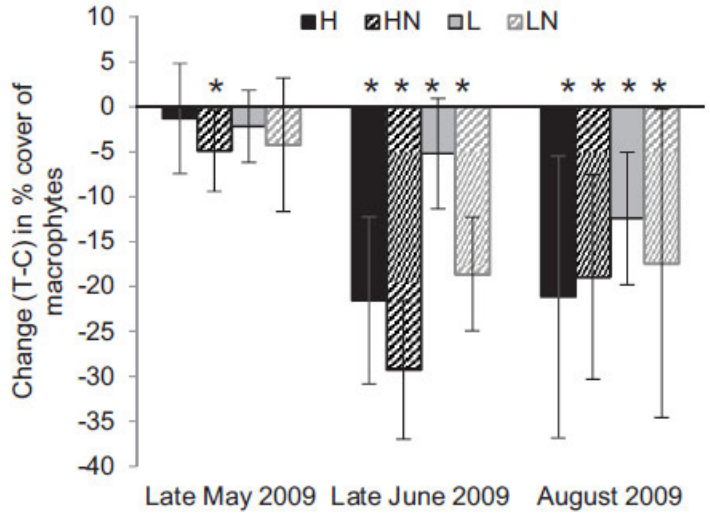
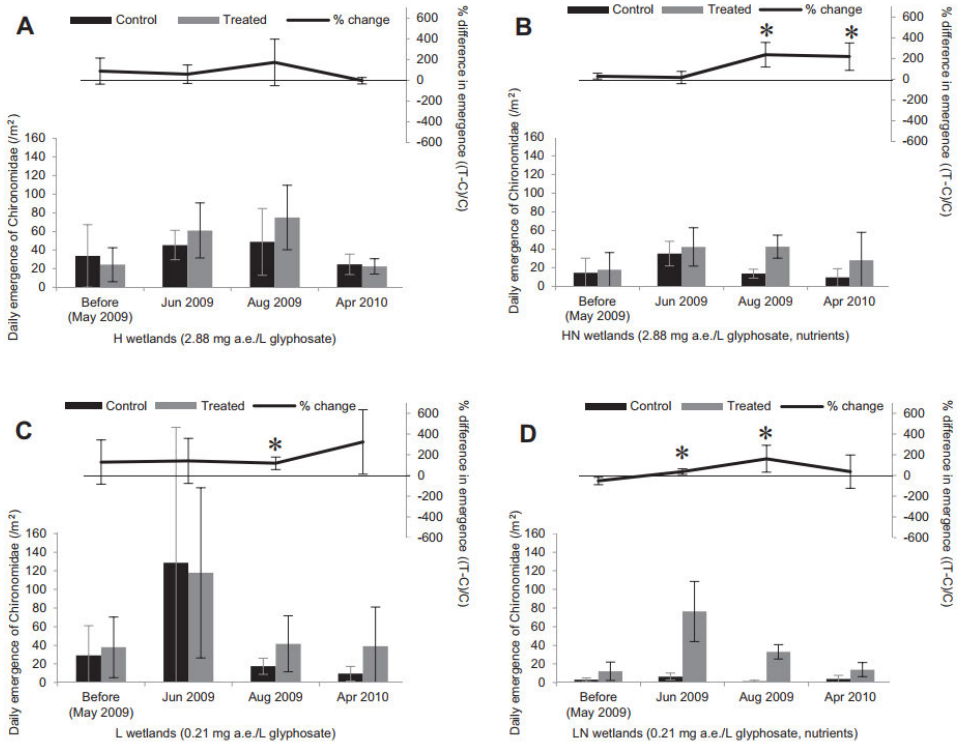


Figure 2. Average differences in the percent cover of macrophytes ± 90% confidence interval; $n = 6$ control and 6 treated experimental units for each treatment category, except in August when only the wetlands containing water could be sampled and H is $n = 3, 3$; HN is $n = 4, 4$; L is $n = 3, 3$; and LN is $n = 3, 3$. H = higher herbicide concentration (2.88 mg acid equivalents/L), HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients. *Significant differences.

Effects on emerging chironomids

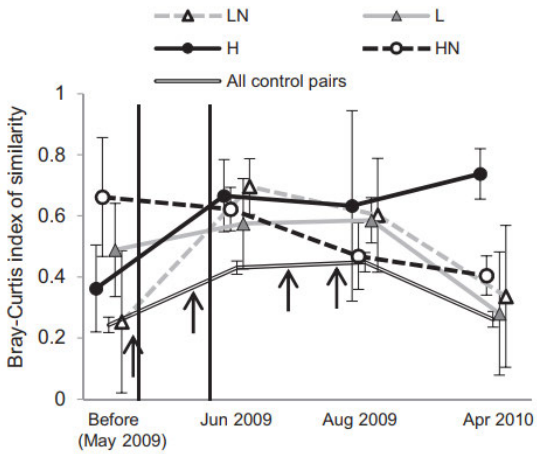
A total of 10 459 chironomids were collected and identified in the present study. Prior to the treatment of wetlands, there was no significant difference in the number of chironomids emerging from the treated sides of wetlands when compared with control sides (+58.1±67.9%, α =0.0005, p=0.174, n=24, 2-tailed, 1-sample t test; Figure 3).

Figure 3. Average daily emergence of Chironomidae from wetlands expressed as average number per square meter (left axis) and the average percent difference (right axis) between sides of wetlands ([(T - C)/C] × 100 ± 90% confidence intervals) in total abundance of all emerging Chironomidae. For each treatment category n = 6 control and 6 treated experimental units, except in August where only the wetlands containing water could be sampled and H is n = 3, 3; HN is n = 4, 4; L is n = 3, 3; and LN is n = 3, 3. Emergence samples were collected in early May were prior to any herbicide application. H = higher herbicide concentration (2.88 mg acid equivalents/L); HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients. * Indicates significant differences.



Average community similarity between wetland halves of all wetlands prior to treatment application was 50.0 ±9.9%. While the average similarity between the sides of each wetland was not particularly high at this time, it was greater than the average similarity among all control sides of wetlands (24.3 ± 2.5% ; Figure 4).

Figure 4. Bray-Curtis index of similarity 90% confidence interval of emerging Chironomidae taxa between the sides of divided wetlands for each treatment. The hollow double line indicates the average similarity among only the control sides of all wetlands, representing the average similarity between independent unaffected wetlands. Similarity ranges from 0 to 1, with 1 = 100% similar communities. H = higher herbicide concentration (2.88 mg acid equivalents/L); HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients.



Little evidence of direct toxic effects of herbicides or herbicides plus nutrients was found on the abundance or assemblage composition of emerging chironomids collected immediately posttreatment in June 2009 (Supplemental Data, Table S1). Only LN ponds showed a significant difference in chironomid emergence on treated sides relative to control sides, with 38 ±27.3% greater average emergence of chironomids on treated versus control sides (2-tailed, 1-sample t test, α =0.0845, p=0.0697; Figure 3). Chironomid assemblage similarity between halves was an average of 19.0% higher than was observed in May 2009, with an average similarity of 64.0 ±5.4% (Figure 4). The average similarity between the control sides of independent wetlands also increased by 18% over the initial pretreatment sampling, to 43.0 ±2.2%. There was a decrease in the proportion of Orthocladiinae chironomids and an increase in the proportion of all other chironomid groups across all wetlands, regardless of treatment type or side of wetland (Figure 5). Wetlands treated with both herbicide and nutrients were not significantly different from wetlands treated with herbicide only in terms of the abundance of emerging midges (H vs HN: p=0.889; and L vs LN: p=0.744; both with N=12, 12, by 2-sample, 2-tailed, independent t tests) as well as the assemblage similarity (H vs HN: p=0.060; L vs LN: p=0.331, α=0.129, n=6, 6, 2-sample, independent, 2-tailed t test). In August 2009, well after the effects of the treatments on the plant community had occurred, there was an increase in chironomid emergence from the treated sides of wetlands relative to their control sides for all treatments except H (H: 173.1±224.1%, n=3, p=0.332; HN: 236.7±118.6%, n=4, p=0.046; L: 117.9 ±61.1%, n=3, p=0.087; LN: 161.5±129.7%, n=3, p=0.177; all by 1-sample, 2-tailed t tests; α=0.244 for H, L, and LN; α=0.166 for HN) (Figure 3).

The mean similarity in the Chironomidae assemblages between sides of each wetland in August was 56.4 ±8.5%, 11.4% higher than before treatment and 7.5% lower than in June (Figure 4). Again, there was an increase in the assemblage similarity in wetlands over the pretreatment levels, and this was contrary to a predicted decline in similarity attributable to any indirect effects of the treatments. While the similarity between wetland halves increased somewhat compared with pretreatment levels, this increase in similarity was only half the average magnitude of the increase in similarity observed between all pairs of control sides of independent wetlands, which increased by 20.5% in August compared with pretreatment (May). Overall, wetlands treated with both herbicide and nutrients were not significantly different from wetlands treated with herbicide only in terms of the abundance of emerging chironomids (H vs HN: p=0.461, n=3, 4, α=0.224; L vs LN: p=0.632, n=3, 3, α=0.251; both by 2-sample, 2-tailed, independent t tests). However, the similarity of the chironomid assemblage in wetlands treated with both herbicide and nutrients was significantly different from that in wetlands treated with herbicide alone in both the higher and lower herbicide additions (H vs HN: p = 0.123, n= 3, 4, α=0.224; L vs LN: p=0.004, n=3, 3, α=0.251; both by 2-sample, 2-tailed, independent t tests). The following spring of 2010, only HN ponds had a significantly higher abundance of emerging chironomids on treated sides relative to control sides (H: -3.5 ±30.3%, α=0.117, p=0.859, n=5; HN: +219.4±131.5%, α=0.084, p=0.041, n=6; L: +323.9 ± 311.4%, α=0.117, p=0.162, n=5; LN: +37.3 ±160.7%, α= 0.084, p=0.718, n=6; all by 2-tailed, 1-sample t tests; Figure 3).

One year posttreatment in most wetlands, the assemblage similarity of Chironomidae was no different from that of the previous spring, indicating possible recovery from any perturbations induced by the

treatments on these organisms (Figure 4). The exception to this was the HN-treated wetland halves, which were on average 13.2 ±27.3% less similar than in the pretreatment data from the same time in 2009 (H: p=0.986; HN: p=0.030; L: p=0.104; LN: p= 0.525; all by 1-tailed, paired t tests, α=0.097, n=6). Although it appears that the predatory Tanypodinae had a higher overall relative abundance in spring 2010 than in spring 2009, this was consistent across all wetland halves and did not appear to be affected by treatment category (Figure 5).

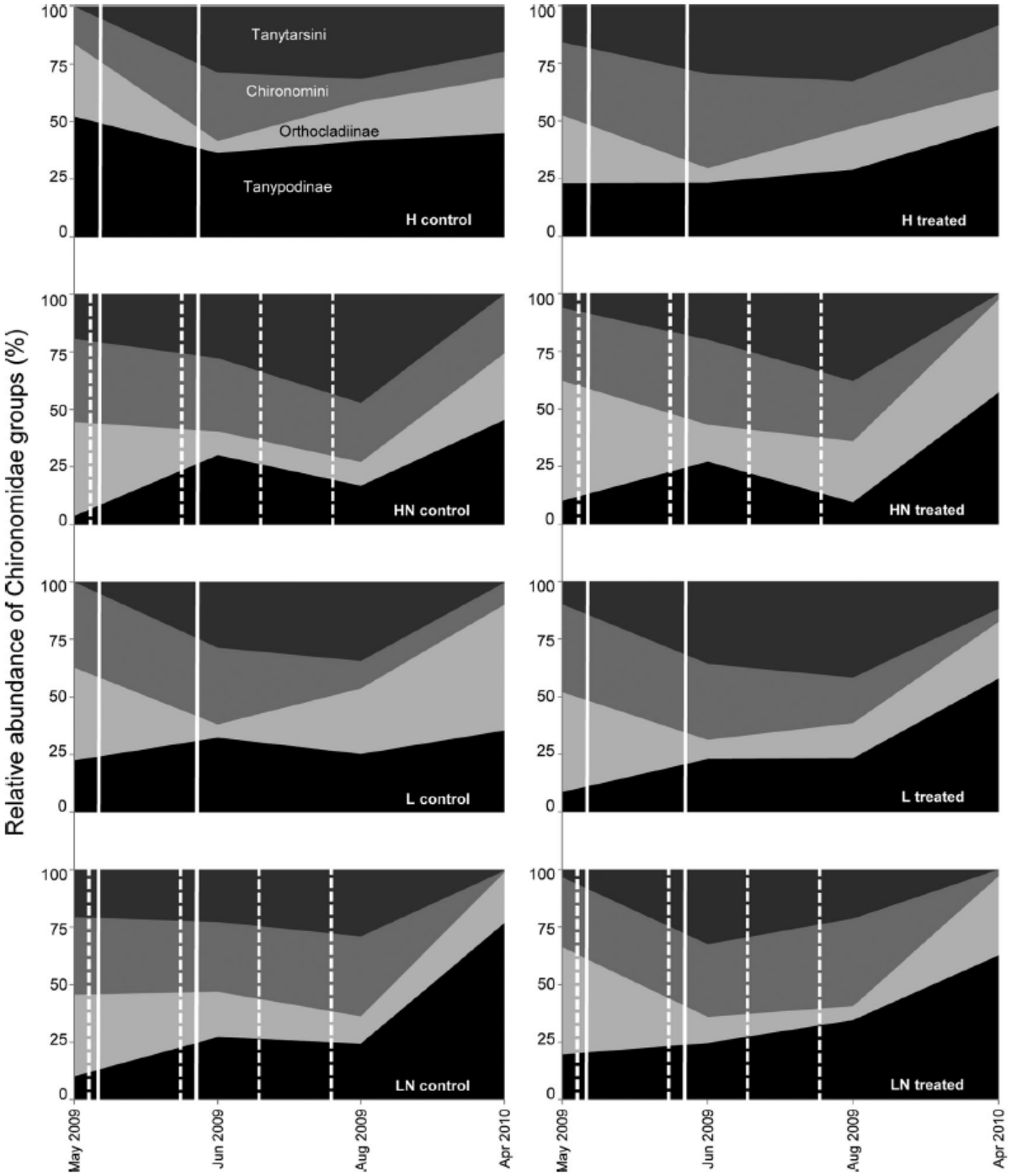


Figure 5. Average relative abundance of major Chironomidae taxa in treated and control sides of wetlands at each sampling time. Vertical solid white lines indicate approximate time of each herbicide application; vertical dashed white lines indicate approximate timing of each nutrient application, relative to sample collection dates. H=higher herbicide concentration (2.88 mg acid equivalents/L); HN=higher herbicide and nutrients; L=lower herbicide concentration (0.21 mg acid equivalents/L); LN=lower herbicide and nutrients.

Effects of macrophyte cover on chironomid emergence

In August 2009, when significant increases in chironomid emergence were observed on treated sides of

wetlands relative to control sides, there was also a significant negative relationship between chironomid emergence and macrophyte cover on all treated sides of wetlands (R^2=0.230, p=0.092, n=13, α=0.145; Figure 6). No relationships between macrophyte cover and chironomid emergence were observed on the control sides of ponds where there were no declines in macrophyte cover; nor were any significant relationships observed in June 2009, when there were no differences in chironomid emergence for either the control or treated sides of wetlands (Supplemental Data, Table S1).

Conclusion

The present study has demonstrated that there was negligible evidence of any negative toxic effects of glyphosate herbicides on the emergence of Chironomidae taxa in the treated wetlands, similar to what has been observed in laboratory and mesocosm studies. The lack of direct toxic effects was likely a result of the lower treatment concentrations used in the study than those that are known to cause acute toxicity, the rapid dissipation of glyphosate from the water column, and the presence of stronger natural drivers of the emergence patterns of chironomid taxa present in the wetlands (e.g., wetland size and hydroperiod, water depth, presence of predators). However, there was evidence of some indirect, longer-term effects, resulting in a higher abundance of emerging midges on treated sides of wetlands. While an increase in the abundance of emerging chironomids may be viewed favorably by some, any change from the natural condition of an ecosystem indicates that the fundamental functioning and structure of the system has been shifted and may be cause for concern.
Emergent plant cover has been hypothesized to play an important role in structuring the chironomid assemblage in wetlands, possibly through habitat cues and physical structure of the pond. The authors were unable to find a strong relationship between the abundance of emerging chironomids and the percent cover of macrophytes in these wetlands under normal conditions. It was only through the application of Roundup WeatherMax and long-term monitoring for indirect effects that we were able to find a relationship between these 2 variables. There may be a more indirect route by which emergent macrophyte structure affects the chironomid assemblage, such as through alteration of algal food sources, but these hypotheses will require investigation. There was also evidence that herbicide impacts on the macrophyte community persisted beyond the year the herbicides were applied (L. Baker, personal observation), whereas chironomid emergence patterns seemed to return to normal. The macrophyte effect on Chironomidae emergence may be present only in taxa that emerge later in the summer, when macrophyte communities are typically more developed. Thus, important areas of further study are to determine the mechanisms by which chironomid emergence is influenced by macrophyte cover and how these mechanisms are affected by herbicide treatment.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The data on the formulation are not relevant for the glyphosate EU renewal (formulation used is not the representative formulation MON 52276). The content of glyphosate in Roundup WeatherMax is not comparable with the representative formulation. Moreover it contains glyphosate in the form of its potassium salt, whereas MON 52276 contains glyphosate isopropyl-ammonium.
Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU).

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Replicated split-wetland experiment was conducted to investigate the effects of 2 nominal concentrations (2.88 mg acid equivalents/L and 0.21 mg acid equivalents/L) of the glyphosate herbicide Roundup WeatherMax, alone or in combination with nutrient additions, on the emergence of Chironomidae (Diptera) before and after herbicide-induced damage to macrophytes.
There were no direct effects of treatment on the structure of the Chironomidae community or on the overall emergence rates. However, after macrophyte cover declined as a result of herbicide application, there were statistically significant increases in emergence in all but the highest herbicide treatment, which had also received no nutrients. There was a negative relationship between chironomid abundance and macrophyte cover on the treated sides of wetlands.
Although direct toxicity of Roundup WeatherMax was not apparent, the authors observed longer-term impacts, suggesting that the indirect effects of this herbicide deserve more consideration when assessing the ecological risk of using herbicides in proximity to wetlands.

Emergence of chironomids is a relevant parameter for the risk assessment. However, RMS notes that measured concentrations of glyphosate were far below those targeted and fastly decreased in the wetlands. Means of 835.57 ±390.68 µg a.e./L (high concentration); 26.69±12.62 µg a.e./L (low concentration).
RMS notes a high variability in daily emergence of chironomidae, within and between groups. As exemple, before treatment, mean emergence was approximately 30 chironomids/m2 in group A, 10 in group B, 30 in group C and 0 in group D*.
*Approximative values as visually estimated based on graphs, no biological data was presented.

RMS notes that statistical analyses in the present study were conducted using an optimal α method to increase statistical power to 88.2%, suggesting a high degree of confidence in the conclusion of no significant effects. It is reported that this method (of Mudge et al, 2012) balances the relative probabilities of type I and II errors to minimize the overall probability of making a wrong conclusion. The objective was to detect a treated mean falling outside of 90% of the predicted control distribution centered on the control mean. Details are available in a supplemental data (not reviewed by RMS).

RMS considers that this study may be relevant to address indirect effects issues. The authors hypothesized that (based on the negative relationship between chironomid emergence rates and plant cover across all treated sides of wetlands in August 2009) the loss of macrophytes from herbicide treatments led to increased chironomid abundance, possibly through some intermediary mechanism such as a loss of predators or increased food amounts.

RMS however notes that significant reduction in plant cover on treated sides of wetlands relative to their control sides following glyphosate herbicide application is not surprising, as reducing plant cover is the intended purpose of glyphosate herbicides (information obtained from Mudge, 2019, also assessed by RMS). The purpose of the additional glyphosate application directly targeting the macrophyte community was to maximize the possibility of indirect impacts of glyphosate herbicides on the invertebrate or amphibian communities through direct effects to the plant community. However, this consistent amount of herbicide applied directly to the plant community on the treated sides of all wetlands was also much higher than the dose received through the different treatment concentrations applied directly to the water's surface.
This study would be then relevant only for aquatic uses where emergent macrophytes are directly exposed.

For regulatory risk assessment, the study is of limited relevance as the exposure of emergent macrophytes (directly sprayed) was considerably higher than expected from a contamination via run-off/drift.
For assessment of indirect effects, the study can be considered relevant and reliable with restriction.

Data point:	CA 9
Report author	Canosa I. S. *et al.*
Report year	2019
Report title	Imbalances in the male reproductive function of the estuarine crab *Neohelice granulata*, caused by glyphosate.
Document No	Ecotoxicology and environmental safety (2019), Vol. 182, pp. 109405
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

The effect of glyphosate, both pure and formulated (Roundup Ultramax®), was evaluated on males of the estuarine crab *Neohelice granulata*, by means of both *in vivo* and *in vitro* assays. The *in vivo* assays comprised the exposure for 30 d to 1 mg/L of the herbicide, until finally assessing weight gain, levels of energy reserves, sperm number *per* spermatophore, proportion of abnormal spermatophores, and sperm viability. At the end of this assay, significant ($p < 0.05$) decrease in weight gain and muscle protein levels was detected by effect of both pure and formulated glyphosate. In spermatophores from the *vas deferens*, a significant ($p < 0.05$) decrease of the sperm count was observed by effect of Roundup, while a significant incidence ($p < 0.05$) of abnormal spermatophores was observed either with glyphosate or with Roundup treatment. No changes were seen in the spermatophore area or in *vas deferens* secretions. Since no sperm mortality was induced by the formulated herbicide, the authors proposed a probable inhibiting effect on spermatogenesis might explain the observed sperm count decrease. In this sense, an *in vitro* assay was designed by incubating testes and *vasa deferentia* with Roundup, in order to corroborate the possible interference of glyphosate with the secretion of the androgenic gland hormone that controls the spermatogenesis, in the presence or absence of the androgenic gland. Although the herbicide *per se* was able to reduce the sperm count to some extent, the increase in the number of spermatozoa/spermatophore produced by the co-incubation with the androgenic gland was completely reverted by the addition of Roundup (1 mg/L of glyphosate a.e.), suggesting that an inhibition on the secretion and/or transduction of the androgenic gland hormone could be taking place.

Materials and methods

Adult males of *N.granulata* were randomly collected in June 2015 at Punta Rasa, the southern edge of Samborombon Bay, Argentina. Males were collected by means of fishery nets, and were identified by their relatively bigger chelipeds, paired gonopods and narrower abdomen, as compared to females. Once in the laboratory, they were acclimated for two weeks to the same environmental conditions and feeding regime used later for the bioassays. All bioassays were conducted in semi-static conditions. Concentrated stock solutions of both Roundup Ultramax® from Monsanto (as soluble granules, 67.9%

w/w of glyphosate as acid equivalent, commercially available) and pure glyphosate (as acid, 99.8% purity; Sigma®, Missouri) were prepared weekly by dissolving the appropriate amount of the chemicals in distilled water. Small aliquots (< 1 mL) were taken from these stock solutions, for diluting in the corresponding test recipient, in order to achieve the desired concentration of herbicide. In order to validate the nominal concentration assayed, water samples (15 mL) were taken at 0 and 72 h, i.e., the period for water replacement in all test containers; after derivatization at pH=9 with 9-fluorenylmethylchloroformate (FMOC-CL), glyphosate concentrations were measured by high pressure liquid chromatography, coupled to a mass spectrophotometry detector Agilent, model VL. A X-SELECT C18 chromatographic column was used. A mixture of MeOH/NH_4 5 mmM/9mM was chosen as mobile phase, with a flow rate of 0.5 mL/min. The test subjects were then observed and evaluated during their pre-reproductive and reproductive periods using both *in vitro* and *in vivo* approaches.

In vivo assays

- *Mortality, molting and energy reserves*

In order to evaluate whether Roundup Ultramax® and its active principle cause alterations in the function of the testis and *vas deferens*, an *in vivo* test was carried out by assigning 10 males to each of the following treatments: Control (dilution water), pure glyphosate at 1 mg/L, or 1 mg/L of glyphosate as acid equivalent (a.e.), in the Roundup Ultramax® formulation. Animals were isolated in glass containers of 1 L capacity, containing 400 mL of saline water (12 g/L, pH: 8) prepared from artificial salts for seawater and dechlorinated tap water (hardness: 80 mg/L as calcium carbonate equivalents). A constant aeration, a temperature of 22 °C, and a photoperiod of 14:10 (L:D) were maintained in all the containers. During the 30 days comprised by the experiment (within the pre-reproductive period), molted and dead crabs were recorded daily; every 72 h the animals were fed *ad libitum* with pelletized food prepared in the laboratory, supplemented with *Elodea* sp, and the water in all the containers was completely replaced (semi-static design). At the beginning and at the end of the assay, the weight of the animals was recorded and weight gain (GP) was calculated as GP = [(final weight - initial)/ initial weight] * 100. At the end of the assay, hemolymph was extracted from the prebranchial sinus for the determination of glycemia by means of the glucose oxidase method. Following, animals were sacrificed by cryoanesthesia, and the hepatopancreas was dissected to estimate the hepatosomatic index as (hepatopancreas wet weight/body weight) × 100. The chelae muscle was also dissected and stored for later determination of energy reserves levels (lipids, proteins, glycogen). At the same time, both the testes and *vasa deferentia* were dissected, fixed in Bouin's solution, and then processed by a routine histological protocol using haematoxylineosin staining.

- *Spermatophores and sperm number*

The following variables were estimated in the histological sections: proportion of abnormal spermatophores, area of the spermatophores and number of spermatozoa *per* spermatophore. Histological methods were employed to get representative sperm samples for all treatment; according to our previous experience in crab species, when electroejaculation is used, remarkable differences in the ejaculated volume among individuals are evident. The Image J 1.51 h program was used for the sperm counting. The photographs were taken with an 8 megapixel digital camera, converted to 8 bits, and the background color was adjusted. The spermatozoa contained in the spermatophores observed in three independent fields were counted. To measure the area of the spermatophores, the perimeter of each spermatophore was marked for the calculation of the area by the program. A total of 30 spermatophores were measured in each crab. In the case of the relative proportion of abnormal spermatophores, the count was performed under the optical microscope, in three independent fields for each individual, recording the number of abnormal spermatophores from the total number observed.

- *Sperm viability*

Since only Roundup caused significant effects on the sperm number, only this product was selected to test the effect on sperm mortality. Therefore a second *in vivo* assay was carried out during the reproductive period, in order to ensure a high sperm content. Ten animals were randomly assigned to each treatment: Control (saline water 12 g/L) and Roundup 1 mg/L (in saline dilution water). The other experimental conditions were similar to those of the first *in vivo* assay. At the end of the assay, the weight of the males were recorded and they were sacrificed by cryoanesthesia. Rapidly, dissection of the vas deferens was performed by cutting with fine-tipped scissors, previously removing the intestine

and hepatopancreas. Once dissected, the left testis and *vas deferens* were weighed to determine the left vasosomatic index as: [(testis wet weight + wet *vas deferens*)/body wet weight) × 100]; once the weight was registered, this material was fixed in Boiun's solution to qualitatively analyze the neutral and acidic glycol-conjugates secretions by PAS/Alcian Blue staining.

The right testis and *vas deferens* were used to determine sperm viability through fluorescence, by staining with Sybr®/propidium iodide probes. The Sybr (Sybr Safe DNA Gel Stain, concentration 10000X, Invitrogen, Thermo Fisher Scientific) has the ability to cross intact cell membranes, while propidium iodide (concentration 1 mg/mL, Thermo Fisher Scientific) displaces Sybr from the dead cells, since it is permeable only through membranes that lost their integrity. Dead cells were observed to fluoresce in red, and the ones alive in green, in a fluorescence microscope (AxioplanZeiss). The count of live and dead sperm was counted out of a total of 500.

In vitro assay
An *in vitro* assay was also carried out during the reproductive period. A total of 24 males were sacrificed by cryoanesthesia, as in previous studies (Medesani et al., 2004), dissecting both testes (T) and *vasa deferentia* (proximal and middle) from each animal, as well as both androgenic glands. The AG of *N. granulata* was located under the stereo microscope. The last portion of the *vas deferens*, to which the AG was attached, was fixed in Bouin's solution, to subsequently perform routine histological processing, in order to confirm its presence.
The treatments were as follows: control; Roundup 1 mg/L; androgenic gland and Roundup 1 mg/L in the presence of the androgenic gland.
At the end of the assay, the testes and *vas deferens* were fixed in Bouin's solution and a routine histological protocol (haematoxylin-eosin staining) was used to process this material, in order to analyze the number of spermatozoa *per* spermatophore.

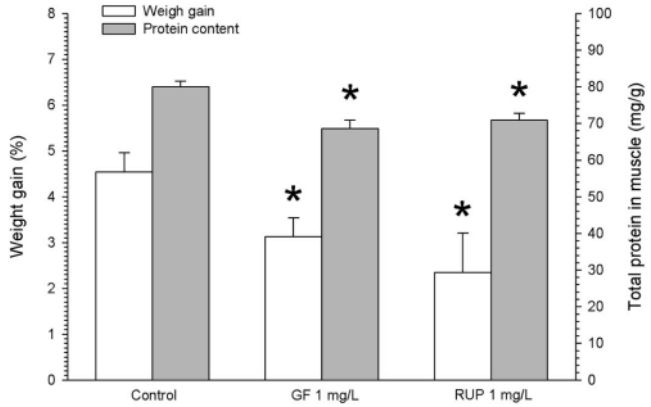
Statistics approach
The proportion of survival and molted males was compared between experimental groups by means of Fisher's exact test (Sokal and Rohlf, 1981). Continuous variables were analyzed by one-way ANOVA, followed by Dunnet's contrast, for comparisons of each treatment with control, and by Tukey's for multiple comparisons. In case that the variance homogeneity was not met, heterogeneity was considered in the data set through the modeling of variances, using the R Studio program; for the particular case of the *in vitro* test, the random block model was incorporated into the varident variance modeling function, through the calculation of the composite symmetry matrix.

Results

In vivo assays
The concentrations of glyphosate, both pure and formulated, determined at 0 and 72 h, averaged 1.27 mg/L ± 0.23 mg/L (ranging from 0.88 to 1.66 mg/L) for pure glyphosate, and 0.92 mg/L ± 0.18 mg/L (ranging from 0.80 to 1.05 mg/L) for Roundup Ultramax®. As mentioned above, all test solutions were completely renewed every 72 h. In the first *in vivo* trial, the percentage of molt and mortality did not show significant differences ($p > 0.05$) between the control and glyphosate-exposed groups. The hepatosomatic index of both groups treated with herbicide was neither different from the control ($p > 0.05$, overall mean 2.35 mg/L ± 0.26).

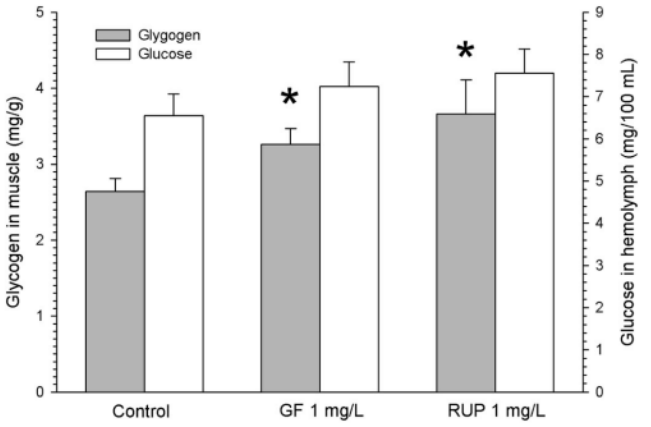
The weight gain was significantly lower ($p < 0.05$) with Roundup 1 mg/L and with glyphosate 1 mg/L, compared to the control (Fig. 1). Although the lipid level in the muscle of the exposed groups was not statistically different from the control level ($p > 0.05$, overall mean = 124.85 ± 5.94 mg/g), both the formulated herbicide and the active principle significantly decreased ($p < 0.05$) the protein level in the muscle, with respect to the control.

Figure 1. Weight gain (%) and protein level in muscle of control animals and exposed to pure glyphosate (GF) or to glyphosate in the Roundup formulation (RUP), at the end of *in vivo* assay for determining histological variables on *N. granulata* males. Asterisks indicate significant differences ($p < 0.05$) with respect to control.



The level of glycogen in the muscle increased significantly ($p < 0.05$) with respect to the control group; at the same time, although no statistically significant differences were observed ($p > 0.05$) in the level of glucose in hemolymph, in both treatments the glycemia tended to increase, with respect to the control (Fig. 2).

Figure 2. Glycogen in muscle and glycemia of control animals and exposed to pure glyphosate (GF) or to glyphosate in the Roundup formulation (RUP), at the end of the in vivo assay for determining histological variables on *N. granulata* males. Asterisks indicate significant differences ($p < 0.05$) with respect to the control.



Regarding the estimation of the number of spermatozoa per spermatophore, a significant reduction ($p < 0.05$) was observed in the group treated with Roundup 1 mg/L with respect to the control, as shown in Table 1.

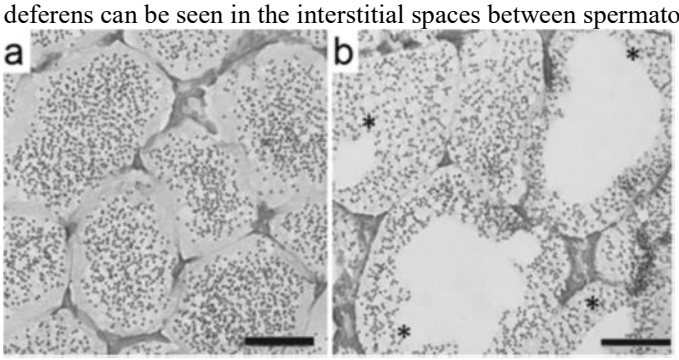
Although pure glyphosate at 1 mg/L showed the same tendency, no significant differences ($p > 0.05$) with control were observed. Concerning the spermatophore area, no significant differences ($p > 0.05$) were seen in between the treatments and the control. However, the percentage of abnormal spermatophores was significantly higher in the treatments with either pure or formulated glyphosate, with respect to the control ($p < 0.05$, Table 1).

Table 1
Percentages of mortality and molting, sperm count *per* spermatophore, spermatophore area percentage of percentage of abnormal spermatophores, at the end of the *in vivo* assay for determining histological variables. Mean values ± standard error are indicated. Initial number of crabs = 10*per* treatment. Asterisks indicate significant differences ($p < 0.05$) with respect to the control; RUP: glyphosate (as acid equivalent) in the Roundup formulation.

Treatment	Mortality (%)	Molt (%)	Sperm count/spermatophore	Spermatophore area (μm^2)	Percentage of abnormal spermatophores
Control	10	0	230.62 ± 18.13	7526.10 ± 2222.93	0.3 ± 0.3
Glyphosate 1 mg/L	0	0	208.88 ± 14.82	8648.58 ± 2573.05	4.6 ± 3.9 *
RUP 1 mg/L	10	10	168.60 ± 15.32 *	7854.69 ± 2581.00	6.5 ± 4.1 *

Fig. 3a shows a vas deferens belonging to a control animal, while Fig. 3b shows a vas deferens corresponding to an animal exposed to Roundup; in the latter, several partially "empty" spermatophores could be observed, with defined concentric areas devoid of sperm, in several cases enlarging the size of the whole structure.

Figure 3. Spermatophores of the middle vas deferens from a control animal (a) and from another specimen exposed to glyphosate in the Roundup, at 1 mg/L (b), at the end of the in vivo assay for determining histological variables. The asterisks indicate the abnormal spermatophores, partially empty of spermatozoa. Several secretions of the vas deferens can be seen in the interstitial spaces between spermatophores. Scale bar: 50 μm.



Concerning sperm viability, although in the treatment with Roundup the sperm mortality was greater than that of control, no statistically significant differences were detected. Regarding the left vasosomatic index, no significant differences were observed ($p > 0.05$) either between the treated or the control groups. The PAS/Alcian blue + secretions observed in the middle *vas deferens* were similar in treated and control animals.

Table 2
Mortality, molting, proportion of dead spermatozoa/total number of spermatozoa and left vasosomatic index, at the end of the in vivo assay for determining sperm viability. Mean values ± standard error are indicated. Initial number of crabs = 10 per treatment; RUP: glyphosate (as acid equivalent) in the Roundup formulation.

Treatment	Mortality (%)	Molt (%)	Proportion of dead spermatozoa	Left vasosomatic index
Control	0	0	4.90 ± 0,80	0.13 ± 0,01
RUP 1 mgL	0	0	5.88 ± 1,27	0.14 ± 0,02

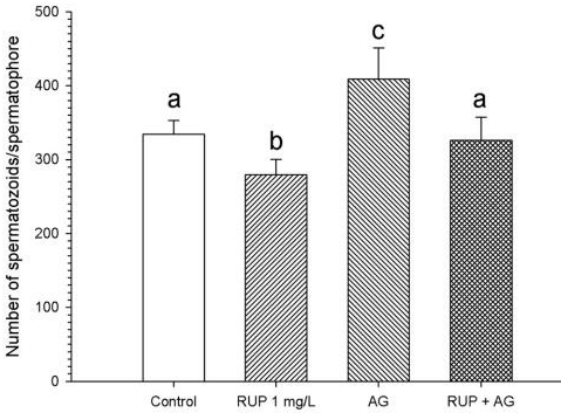
In vitro assay

Each portion of the wall of the *vas deferens* could be discerned by the histological differences exhibited along its length. The proximal vas deferens was characterized by a columnar epithelium with abundant secretory cells. The middle vas deferens showed a much thinner simple epithelium as previously described by López Greco (1997), while the distal vas deferens was easily recognizable by the abundant lateral translucent caeca. The androgenic gland, a paired organ, was observed as a white-opalescent elongated structure, approximately 0.7mm long and 50 μm wide, weakly attached to the subterminal portion of the distal vas deferens, between the caeca and the coxa of the fifth pereiopod. In this in vitro assay, a significant reduction ($p < 0.05$) in the number of spermatozoa per spermatophore was observed in the treatment that only received 1 mg/L of Roundup, as compared to control. In turn, in the treatment that only received androgenic gland, there was a significant increase ($p < 0.05$) of this variable over the

control levels. However, in the treatment with the herbicide formulated in the presence of the androgenic gland, the number of spermatozoa/spermatophore was similar to the control ($p > 0.05$).

In this assay, neither the presence of spermatophores with abnormalities, nor differences in the area of the spermatophores were evidenced. Besides, no changes were observed between controls and exposed to Roundup, in relation to the secretions from the *vas deferens*.

Figure 4. Total sperm count per spermatophore in the control, glyphosate in the Roundup (RUP) at 1 mg/L, androgenic gland (AG), and RUP at 1 mg/L + androgenic gland (RUP + AG) treatments, at the end of the *in vitro* assay. Different letters indicate significant differences ($p < 0.05$) between the experimental groups.



Conclusion

The authors conclude that glyphosate, both pure and formulated, is able to affect the reproductive performance of male crabs after 1-month exposure period to 1 mg/L of the active ingredient, by producing abnormal spermatophores and a reduction in sperm count. This latter effect would be due, at least in part, to the interference of the herbicide with the endocrine control exerted by the androgenic gland. Furthermore, the analysis of different variables aimed at estimating the sperm quality of male crabs exposed to glyphosate would be a useful ecotoxicological approach to predict a possible short-term effect on egg production at a population level.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested (Roundup Ultramax, 67.9% w/w of glyphosate a.e., formulated using potassium salt) is not the representative formulation for the glyphosate EU renewal. Additionaly, this Argentinian study can be difficult to extrapolate to EU, local native species - blue crab, geo-climatic properties, land-uses and agricultural practices differs from EU. Moreover, the exposure profile and concentrations tested are not considered realistic for an EU level evaluation.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Males of the estuarine crab (Neohelice granulate) were exposed to both pure glyphosate and a glyphosate formulation (Roundup Ultramax, containing glyphosate) by means of both in vivo and in vitro assays.

In vivo assay
The in vivo assays comprised the exposure for 30 d to 1 mg/L of the herbicide, until finally assessing weight gain, levels of energy reserves, sperm number per spermatophore, proportion of abnormal spermatophores, and sperm viability.

Results:
- significant ($p < 0.05$) decrease in weight gain and muscle protein levels was detected by effect of both pure and formulated glyphosate.

RMS notes level of lipids were not affected by exposure to pure glyphosate or Roundup, the protein level in the muscle showed a significant decrease in agreement with the decrease in weight gain. These may be indicative of a glyphosate toxic effect (as suggested by the authors).
- significant ($p < 0.05$) decrease of the sperm count was observed by effect of Roundup,
- significant incidence ($p < 0.05$) of abnormal spermatophores was observed either with glyphosate or with Roundup treatment. RMS however notes important variability on these results (based on standard deviation, no biological data presented).

RMS notes that the number of spermatozoa per spermatophore was significantly lower in the group treated with Roundup, compared to control; although this significant decrease was not exhibited with pure glyphosate, a similar trend was observed. Consequently, the coadjuvants present in the commercial formulation, would enhance in some way the incidence of this pathology, either by facilitating the entry of glyphosate into the tissues and/or due to its intrinsic toxicity.
- No changes were seen in the spermatophore area or in vas deferens secretions.
- Since no sperm mortality was induced by the formulated herbicide, the authors proposed a probable inhibiting effect on spermatogenesis might explain the observed sperm count decrease.

In vitro assay
An in vitro assay was designed by incubating testes and vasa deferentia with Roundup, in order to corroborate the possible interference of glyphosate (in Roundup) with the secretion of the androgenic gland hormone that controls the spermatogenesis, in the presence or absence of the androgenic gland.
Results:
- the herbicide per se was able to reduce the sperm count to some extent,
- the increase in the number of spermatozoa/spermatophore produced by the co-incubation with the androgenic gland was completely reverted by the addition of Roundup (1 mg/L of glyphosate a.e.), suggesting that an inhibition on the secretion and/or transduction of the androgenic gland hormone could be taking place.

This study originates from the same lab that produced the following study:
Avigliano L. et al., 2014; Effects of glyphosate on egg incubation, larvae hatching, and ovarian rematuration in the estuarine crab Neohelice granulate.
Avigliano L. et al., 2014 stated that based on a comparison of number of hatched larvae per female between Roundup Ultramax (clear embryonic mortality) and glyphosate (no significant increase of mortality) at equivalent concentration, Roundup compounds other than glyphosate may be responsible for the embryonic mortality.
RMS questions the relevance of the data obtained on Roundup Ultramax as no discrimination between glyphosate and other compounds is feasible.
Only the results available for the pure form of glyphosate are deemed relevant by RMS. RMS considers that results obtained with the formulation only (i.e. in vitro assay) should be considered with caution and should not be used to derive critical endpoint.

Overall:
For glyphosate alone, decrease in weight gain and muscle protein levels and higher incidence of abnormal spermatophores may be attributed to glyphosate at the concentration of 1.27 mg/L.

Reliability assessment:
The study did not follow standardized guideline.
Concentrations were analytically verified.
Only one concentration (1 mg/L) was assayed and no mortality was observed (out of 10 males), so the MTC cannot be determined.
Concerning the potential impact of using wild-caught organisms, particularly regarding potential effects on the endocrine system from prior exposure to other substances, it is mentioned in the report that the specimens were randomly collected at the southern edge of Samborombón Bay, at the mouth of the Rio de la Plata estuary, Argentina. The contamination level cannot be verified by RMS, it is only stated that very scarce information about glyphosate environmental levels has been published, with reported values between 0.1 mg/L and 0.7 mg/L in water and between 0.5 mg/kg and 5 mg/kg in sediments. However, the report also states (in the introduction) that heavy charge of herbicides and other pesticides is carried by several rivers and channels that cross extensive agricultural areas and finally reach the Samborombón Bay (Comisión Administrativa del Río de La Plata 1990). RMS then cannot discard the presence of other toxicants in this estuary.

Overall, it is not possible to relate the observed effects to an endocrine mode of action (MTC not defined).

The study is considered reliable with restrictions (for effects on bodyweight gain, not reliable for endocrine properties). RMS however notes that only bodyweight gain is reported not bodyweight itself. So the magnitude of the effect is uncertain and potentially low. Effects on abnormal spermatophores are considered of low reliability due to variability on the results.

The study is considered relevant and reliable with restrictions (for effect on bodyweight gain). Results for ED assessment purpose are unreliable.

Data point:	CA 9
Report author	Baker L. F. *et al.*
Report year	2016
Report title	The combined influence of two agricultural contaminants on natural communities of phytoplankton and zooplankton
Document No	Ecotoxicology, (2016) Vol. 25, No. 5, pp. 1021-32
Guidelines followed in study	None
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by title/abstract

Full summary of the study according to OECD format

By examining changes in the phytoplankton and zooplankton communities of shallow, partitioned wetlands over a 5 month period, the researchers assessed the potential for direct and indirect effects of the glyphosate-based herbicide, Roundup WeatherMax applied at the maximum label rate, both in isolation and in a mixture with nutrients (from fertilizers). The co-application of herbicide and nutrients resulted in an immediate but transient decline in dietary quality of phytoplankton (8.3 % decline in edible carbon content/L) and zooplankton community similarity (27 % decline in similarity and loss of three taxa), whereas these effects were not evident in wetlands treated only with the herbicide. Indirect effects of the herbicide-nutrient mixture were evident in mid-summer, when glyphosate residues were no longer detectable in surface water. Zooplankton abundance tripled, and zooplankton taxa richness increased by an average of four taxa in the herbicide and nutrient treated wetlands. The lack of significant toxicity of Roundup WeatherMax alone, as well as the observation of delayed interactive or indirect effects of the mixture of herbicide and nutrients attest to the value of manipulative field experiments as part of a comprehensive, tiered approach to risk assessments in ecotoxicology.

Materials and methods

Test substances: Roundup WeatherMax

Site description and barrier design
Wetlands used in this study were located in the Long-term Experimental Wetlands Area, a 4-km^2 site on Canadian Forces Base Gagetown, approximately 60 km northwest of Saint John, NB, Canada (66°29'59.02"W, 45°40'48.62"N).
Wetlands formed in depressions and next to windrows immediately after mechanical clearing of the land in 1997–1998. The area was not treated with any chemical pesticides prior to this experiment. Wetlands were generally shallow (21–90 cm deep at high water periods), fishless and unstratified. Barriers were used to divide the wetlands, which were composed of 30 mil (0.76 mm) opaque black, high-density polyethylene (HDPE) geomembrane (Poly-Flex Inc., Geomembrane Lining Systems, Grand Prairie, TX, USA). The barriers were approximately 1 m in height and had sealed pockets filled with crushed gravel along the bottom to anchor them into the sediments. These barriers, installed in August 2008, stretched the entire length of the wetland and extended beyond the high water mark (Fig. 1).

Fig. 1 HDPE barrier dividing an experimental wetland

Chemical application and quantification
The experimental design and quantification of chemical concentrations has been previously described (Baker et al. 2014). Briefly, in each of the six divided wetlands, one randomly selected half was hand-sprayed using a backpack sprayer with the agricultural-use herbicide Roundup WeatherMax (Monsanto Company, Creve Coeur, MO, USA). Two separate herbicide applications were conducted on May 15–16, and June 9–10, 2009, to mimic the timing of agricultural weed control applications in the region. For this study, we examined the effects of a predicted maximum environmental aqueous concentration of 2.88 mg a.e./L of glyphosate on six wetlands. This predicted concentration was calculated as the concentration that could result from direct overspray of a 15 cm deep wetland at the maximum label

application rate of 4.32 kg a.e./ha with no interception by emergent macrophytes. This treatment level is termed ‘‘higher’’ (H) glyphosate concentration to maintain consistency with treatments applied in other experimental wetlands not described here (see Baker et al. 2014; Edge et al. 2014).
Additionally, solutions of nutrients were applied to the herbicide-sprayed side of three of the six wetlands (referred to as ‘‘HN’’ treatments). The nutrient solution was generated from salts of nutrients typically used in fertilizers (technical grade ammonium nitrate and phosphoric acid, Fisher Scientific), and applied on each of May 14, May 29, July 3 and August 19, 2009. Nutrients were added to each wetland with the objective of increasing the aqueous phosphorus (measured as total phosphorus (TP)) to 0.1 mg/L (i.e. eutrophic; Wetzel 2001) over the background concentrations measured in 2008, and aqueous nitrogen (measured as total Kjeldahl Nitrogen (TKN)) was added to maintain the natural TKN:TP ratio, so as to prevent a shift in which of N or P was the limiting nutrient naturally. The full experimental design here in consisted of 3 H wetland halves and 3 HN wetland halves, each with a paired untreated half (12 experimental units in total, from six bisected wetlands).
Water samples were collected from all experimental units every 2 weeks through the course of the summer for nutrient analysis. Glyphosate concentrations were measured in water samples collected from both sides of each wetland on days 1, 3 and 7 after the first round of spraying, and on days 0, 3 and 7 after the second round of spraying.
Control sides of wetlands were verified as having glyphosate concentrations below detection limits (3.54 µg/L), except in three instances where the concentration was 8 µg/L in Ag-07 (both applications) and Ag-21 (second application), although it was not considered to be a concern as these concentrations were very low, less than 0.2 % of the target concentration. Quantitation methods, quality assurance, and discussion of results are presented in detail in Baker et al. (2014) and in Edge et al. (2014). Concentrations pertinent to this study have been discussed herein.

Plankton sampling and enumeration
Depth-integrated plankton samples were collected from each side of each wetland simultaneously using a 20 µm mesh, weighted, student plankton net (15 cm diameter opening, Dynamic Aqua-Supply Ltd., Surrey, BC, Canada). A set of pre-exposure samples were collected 3–5 days before applications (‘‘before’’ samples). The first to sixth sampling periods occurred on days 1, 5, 10, 16, 21, and 23 after the first herbicide treatment. The 7th–14th sampling periods occurred on days 1, 6, 10/11, 15, 20, 30, 44/45, and 90 after the second herbicide treatment. Due to temporary dry-downs samples could not be collected during the 6th and 13th sampling periods in one replicate of the herbicide and nutrient treatment and the 13th sampling period of one replicate of the herbicide alone treatment. Sub-samples collected from five, flagged, permanent sampling stations distributed within each half of each wetland were combined and preserved with 5 % Lugol’s solution in the field. Volume of the sampled column of water was calculated as a cylinder using the diameter of the plankton net and the known total depth sampled on each side for each day.
Zooplankton in a sub-sample from each sample were enumerated and identified to species level where possible, or to the lowest practical taxonomic level (Ward and Whipple 1945; Thorp and Covich 2001) using a Bogorov counting chamber (WildcoTM, Yulee, FL, USA) under dissecting microscope. From this, zooplankton abundance, richness and community similarity were calculated. Zooplankton abundance was a tally of all zooplankton individuals across all taxa on a particular date for each wetland side. Zooplankton richness was a tally of unique taxa on a particular date for each wetland side. We used the additive inverse of the Bray–Curtis dissimilarity index (Bray and Curtis 1957) as a semi-quantitative measure of the similarity in zooplankton community composition between sides of the wetlands. The average similarity between only the control sides of all wetlands was used as a baseline comparison of the degree of similarity among wetlands to be expected at the landscape level.
For phytoplankton endpoints, aliquots from the same samples used to enumerate zooplankton were placed in a 0.1 mL nanoplankton counting chamber (PhycoTech Inc., St. Joseph, MI, USA). A transect of eight images was taken across the middle of the plankton chamber (image dimensions: 2358 x 1768 µm each). Images were converted to grayscale and analyzed with Image J software (Rasband Rasband 1997–2012). The default thresholding tool was used to separate the dark phytoplankton particles from the lighter background. The area, width and maximum length of each phytoplankton cell was calculated using the Image J particle analyzer tool. Total cell volume was estimated by multiplying the area of each cell in the image by the smaller dimension of the smallest rectangle that could enclose the cell (or by

520 µm, the thickness of the chamber, if the smaller dimension of the smallest rectangle that could enclose the particle was greater than 520 µm). Cells were assumed to lie with the largest cross-sectional area parallel (i.e. lie flat) to the bottom surface of the counting chamber; the smaller dimension of the smallest rectangle that could enclose the cell represents the maximum possible height of each cell. This results in an estimate of the maximum possible volume of phytoplankton cells within each sample, an approximation (but likely somewhat of an overestimation) of the true phytoplankton cell volume in each sample. The dietary quality of phytoplankton was calculated as the total volume of edible carbon within only those phytoplankton cells having the largest diameter 40 µm (which is generally considered to be within the gape size of zooplankton) using the following formula: Edible carbon (pg C) = 0.1204 x (volume (µl) of <40 µm cells)$^{1.051}$ (Rocha and Duncan 1985).

Statistical analysis

In this study, experimental units were wetland halves (n = 12). To maintain the power of the paired-wetland design, all endpoints except zooplankton community similarity and richness are expressed as a baseline standardized (value of control side) difference between what was observed in the control side subtracted from the value measured in the treated sides ((Ti-Ci)/ Ci) x 100. One-tailed, paired t-tests were used to determine the significance of declines in zooplankton and phytoplankton endpoints in the sampling periods immediately following both applications of herbicides (May 16/17 and June 10/11), as it is expected that herbicide toxicity would manifest as a loss in the relative amounts of phytoplankton and zooplankton. Two tailed, paired t-tests were used to determine if there significant differences in phytoplankton and zooplankton endpoints in response to visible damage to macrophytes resulting from herbicide applications in the first sampling time point beyond which such damage had been observed (July 9/10; see Baker et al. 2014 for details) as well as at the end of the summer (September 7/8). All tests for differences in the Bray–Curtis index of similarity were conducted as one-tailed tests in comparison to the pretreatment levels of similarity (baseline) where all expected changes in community composition should present as the sides of wetlands becoming less similar. All data are presented as the mean ± 90 % confidence intervals in the text and graphs.

To determine time-dependent effects of each treatment (H and HN) through the summer on the zooplankton community structure, the matrix of $\log_{10}(x + 1)$ transformed absolute abundance of zooplankton taxa was analyzed using the constrained multivariate ordination technique of partial redundancy analysis (pRDA) in the vegan package (Oksanen et al. 2015) in R (R Development Core Team 2012). Explanatory variables were treatment, time, and treatment regime (treatment x sampling time), where the effects of wetland and time were coded as dummy covariates such that these were ‘‘partialled out’’ similar to a repeated measures ANOVA with wetland and time as blocking factors. Significance of the model was tested by permutation (n = 199). This technique emphasizes the percentage change in abundance of taxa in the treatments relative to controls, independent from absolute abundance (van den Brink and ter Braak 1999). For all analyses, a compromise between Type I and II errors was reached by choosing the α level that minimizes the average of α and β at a given critical effect size, accomplished by an iterative examination of β (through power analysis) over a range of α-levels (Mudge et al. 2012). Type I and II errors were considered to have equal costs, as there is no substantive evidence to indicate that either type of error was more serious/costly in these circumstances. The mean of a treatment variable falling outside 90 % of the predicted control distribution centered on the control mean was deemed to be a critical effect size (CES) to be detected, should it exist (Munkittrick et al. 2009). For a two-tailed test this corresponded to a CES of 1.64 standard deviations (SD) of the data, and 1.28 SD of the data was used for one-tailed tests. When there was no clear directional hypothesis the CES associated with a two-tailed test was used. For each test, equal prior probabilities of the null or alternative hypotheses being true were assumed. Optimal α and associated betas and omegas (ω = average chance of making a wrong conclusion) for each sample size were calculated using R with code from Mudge et al. (2012).

Results

Nutrient and glyphosate concentrations

Measures of nutrient concentrations in wetland water were lower than intended target concentrations after applications of nutrients began. Ammonium (NH_4+) concentrations varied substantially throughout the course of the

experiment, whereas TP concentrations remained relatively constant following the first nutrient addition. On average, the NH4+ concentration in treated wetland halves was 0.02 ± 0.03 mg/L higher than controls, and TP was 0.05 ± 0.01 mg/L higher than controls. Glyphosate residues in the water column of the treated sides of experimental wetlands declined rapidly and were undetectable beyond 7 days post initial herbicide application. After this first herbicide application, the glyphosate concentration in the treated sides of all wetlands in the present study (n = 6) was 799 ± 644 µg a.e./L at 1 day post-application, 108 ± 112 µg a.e./L at 3 days post-application and 13 ± 11 µg a.e./L at 7 days post application. Despite having the same target glyphosate concentrations, glyphosate residues appeared (not statistically significant) to decline more slowly in the herbicide with nutrients-treated wetlands than the herbicide alone-treated wetlands, where the herbicide and nutrient wetlands had higher glyphosate concentrations of 1173.1 ± 1256.5 µg a.e./L on Day 1 ($p = 0.398$, $n = 3,3$, $\alpha = 0.244$, paired two-tailed t-test) and 195.1 ± 205.1 µg a.e./L on Day 3 ($p = 0.239$, $n = 3,3$, $\alpha = 0.244$, paired two-tailed t-test) than was observed in the herbicide alone-treated wetlands had glyphosate concentrations of 424.5 ± 343 µg a.e./L on Day 1 and 22.3 ± 18.3 µg a.e./L on Day 3. It was examined whether dissipation rates of the two treatments could be related to differences in natural features of the wetlands including water depth and volume (of the treated half of wetlands only), or due to microbial decomposition capacity (as background microbial respiration rates). Post hoc linear regression analysis demonstrated that the maximum estimated water volume explained only a small portion of the variation in glyphosate concentrations ($R^2 = 0.14$, $p = 0.050$, $n = 12$) where larger wetlands halves had slower glyphosate removal rates. Microbial respiration rates also explained a very small amount of dissipation rates ($R^2 = 0.043$, $p = 0.0002$, $n = 11$), with higher respiration rates leading to more rapid glyphosate removal. Ultimately, glyphosate removal rates were not well described by these factors. In water samples collected after the second herbicide application, glyphosate residues in treated sides of all wetlands (n = 6) were measured at 215 ± 244 µg a.e./L and 40 ± 38 µg a.e./L, at 3 and 7 days post-application, respectively. Based on these concentrations the half-life of glyphosate in the water of the treated wetlands was calculated to be approximately 1 day for each application period.

Environmental conditions: Not stated

Pre-treatment characteristics of wetland plankton communities

Phytoplankton cell volumes ranged from 4.7 to 2608.0 µL/ L and averaged 94.7 ± 0.06 µL/L during the pre-treatment period. Phytoplankton dietary quality (measured as edible carbon) ranged from 0.003 to 0.528 pg/L of wetland water, with an average of 0.090 pg/L. Generally phytoplankton amounts and quality (edible carbon content) were highly variable over the summer. Zooplankton abundance ranged from 3 to 331 organisms/L, with an average 44.2 organisms/L prior to chemical treatments across all wetland halves. Samples contained between 3 and 12 zooplankton taxa each with a total of 18 taxa found. Cyclopoida sp. adults and Copepoda nauplii dominated, followed by the cladoceran, Chydorus sphaericus. The similarity of zooplankton community composition was on average 59.0 ± 1 % (n = 6) between control and treated sides prior to chemical applications, which was similar to the natural levels of Bray–Curtis similarity measured in other studies (Cottenie et al. 2003; Hunt et al. 2008). The average community similarity of zooplankton between the paired halves of a wetland was 22.6 % higher than the average of the similarity found between independent wetlands (36.4 ± 11 %), suggesting that the paired wetland half design of this experiment significantly improved the overall power of the

experimental design.

Short-term effects on plankton communities

One day after the first application of the herbicide to the treated sides of the nutrient-treated wetlands (HN treatment) there was a significant decline in phytoplankton quality (-8.3 ± 12.4 %, p = 0.154, n = 3, α = 0.212 one-tailed paired t-test, Fig. 2b) and in zooplankton abundance of 37.7 ± 23.5 % (Fig. 3a), or an average loss of approximately 14 zooplankters/L (p = 0.060, n = 3, α = 0.212, one-tailed paired t-test). This same treatment group also experienced a significant decline in zooplankton taxa richness, with an average loss of 2.7 ± 2.9 taxa (Fig. 3b), (p = 0.135, n = 3, α = 0.212, one-tailed paired t-test). This also led to a decline in the overall similarity of the herbicide and nutrient treated zooplankton communities of 27.8 ± 29.2 % (p = 0.129, n = 3, α = 0.212, paired one-tailed t-test) (Fig. 3c). In contrast, during this initial response period for the herbicide alone treatment there was only a small, but significant decline in zooplankton richness of 1.7 ± 0.55 taxa (p = 0.019, n = 3, α = 0.212 paired one-tailed t-test), with no attendant declines in zooplankton abundance or community similarity, nor in phytoplankton quality or amounts (Fig. 2 and 3, Online Resource 1). As shown in Fig. 3, one day prior to the second herbicide application the zooplankton communities of all treated wetlands appeared to have recovered to approximately pre-treatment levels of abundance (H p = 0.519, HN p = 0.821), richness (H p = 0.500, HN p = 0.250) and community similarity (H p = 0.243, HN p = 0.327). Unlike after the first application, one day after the second application of herbicide, there were no significant changes observed in phytoplankton or zooplankton endpoints in the herbicide and nutrient treated wetlands (Fig. 2 and 3, and Online Resource 1). For the herbicide only treated wetlands, phytoplankton abundance and quality appeared to decline, but not significantly. However, the richness of zooplankton in the herbicide alone treated wetlands was reduced by an average of 2.7 ± 0.6 taxa compared to controls (p = 0.008, n = 3, α = 0.212, one-tailed paired t-test). Zooplankton abundance and community similarity were not significantly different between treatment and control halves as a result of the second herbicide application for either treatment.

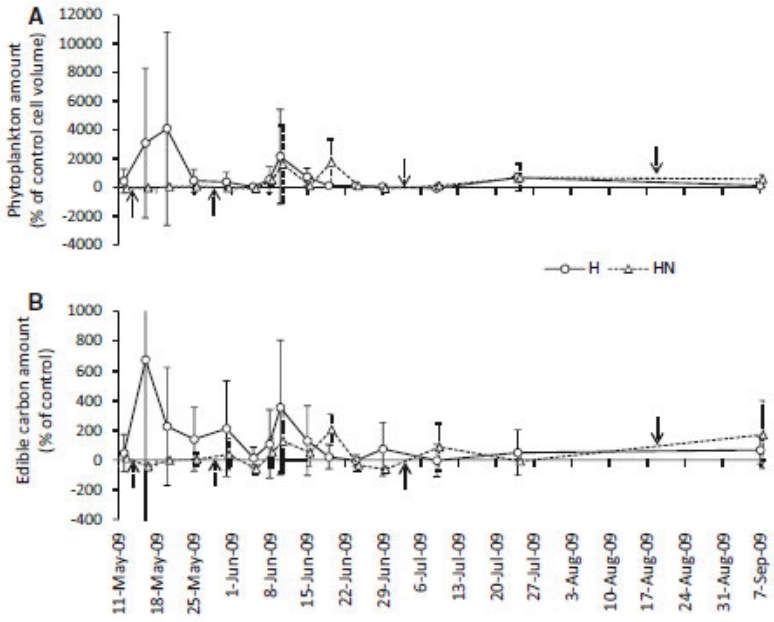


Fig. 2 Change (±90 % CI), as percentage of the control, in phytoplankton **a** cell volume and **b** edible carbon in the treated sides of wetlands, (H: n = 3, HN: n = 3). H = treatments with 2.88 mg a.e./L of Roundup WeatherMax® on the dates indicated by the *vertical lines*. HN = identical herbicide treatment regime with the addition of nutrients to wetlands on dates indicated by *arrows*

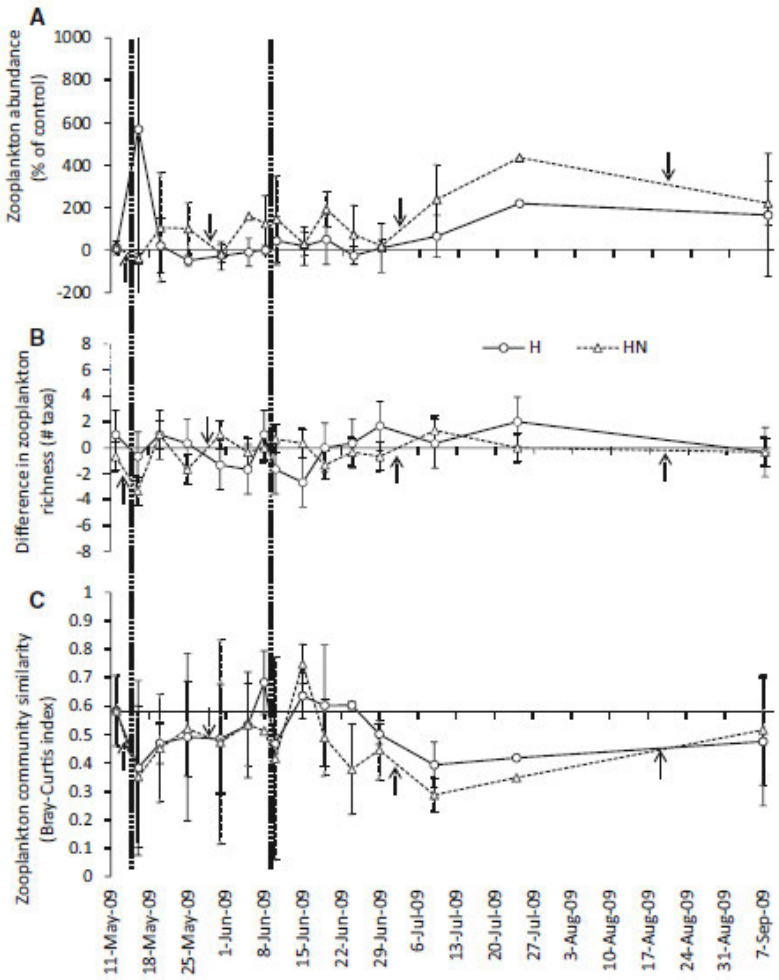


Fig. 3 Differences (±90 % CI) in zooplankton **a** abundance, as a percentage of control; **b** richness (treatment–control) and **c** the average community similarity between the treated sides of wetlands compared to respective control sides (H: n = 3, HN: n = 3). H = treatments with 2.88 mg a.e./L of Roundup WeatherMax® on the dates indicated by the *vertical lines*. HN = identical herbicide treatment regime with the addition of nutrients to wetlands on dates indicated by the *arrows*

Longer-term effects on plankton communities

After macrophyte declines were first observed on June 24–25 (Baker et al. 2014), which was 10 days beyond when glyphosate was no longer measurable in the water column, changes in the zooplankton (Fig. 3a)—but not phytoplankton (Fig. 2)—community were observed. By July, the herbicide and nutrient treated wetlands showed no significant changes in algal abundance or quality. However, zooplankton communities in this treatment became less similar, by as much as 34.1 ± 2.0 % on July 9/10 ($p = 0.001$, $n = 3$, $\alpha = 0.212$, paired one-tailed t-test), compared to pre-treatment levels, an effect which persisted for over 6 weeks, past the July 24th (period 11) sampling date (Fig. 3a). This change was much larger than natural background variability where there was a minimal change in community composition in control sides of wetlands (similarity among the control halves of wetlands had not otherwise changed—on average 7.2 ± 7.6 % below pretreatment levels). At this same time the abundance and richness of zooplankton in treatment versus control halves of the herbicide and nutrient treated wetlands increased by 238.6 ± 163.7 % ($p = 0.139$, $n = 3$, $\alpha = 0.244$ paired, two-tailed t-test) and by 1.33 ± 2.19 taxa ($p = 0.213$, $n = 3$, $\alpha = 0.244$, paired two-tailed t-test), respectively. Herbicide alone treated wetlands showed variable but no significant differences in phytoplankton amounts and quality. When compared to the herbicide and nutrient treated wetlands, all zooplankton endpoints in the herbicide only treated wetlands showed similar but non-significant trends.

Late in the summer (Sep 7/8 sampling period) there was also evidence of ongoing effects of the herbicide and nutrient treatments on phytoplankton (Fig. 2). There was an increase in phytoplankton amounts by 640.7 ± 295.2 % compared to controls ($p = 0.182$, $n = 3$, $\alpha = 0.244$, paired two-tailed t-test). The trend of significantly higher abundance of zooplankton in herbicide and nutrient treated wetlands continued through to the end of the summer, where there was, on average, 221.4 ± 102.7 % more zooplankton in the affected sides of herbicide and nutrient treated wetlands than the controls ($p = 0.071$, $n = 3$, $\alpha = 0.244$, paired two-tailed t-tests). However, the richness and similarity of zooplankton communities had returned to approximately pre-treatment levels by this date.

Phytoplankton amounts and quality in herbicide alone treated wetlands were variable, but ultimately

were not significantly affected. Zooplankton communities of wetlands that had been treated with only herbicides showed responses similar in direction, but not magnitude to those of the herbicide and nutrient treated wetlands.
An examination of the cumulative effects of treatments on zooplankton community composition in the wetlands over the summer showed that treatment regime (treatment x sampling date) explained 8.7 % of the variability in the zooplankton community composition data, and treatment category explained 0.7 %. In contrast the partialled covariates of sampling date and wetland explained 35.7 % of the variability where 54.9 % of the variation in zooplankton community was not explained at all by this model pRDA. Zooplankton taxa did not strongly load on the first two axes of the model (species weight of <0.5), which is logical considering the lack of significant overlap in species composition among all wetlands and, as such, the researchers could not comment on effects on individual taxa. The model was not statistically significant ($p = 0.680$, by permutation $n = 199$). The effects (across wetlands) of treatment regime (treatment x sampling time) of herbicides alone and herbicide + nutrient treatments had inconsistent effects on the zooplankton community, which is consistent with the data described herein showing different effects in the short term versus long term. This result suggests that experimental treatments on the whole were less important in determining zooplankton community composition than wetland-specific factors or time of year.

Conclusion

A worst-case contamination of wetlands with the herbicide Roundup WeatherMax in combination with fertilizer nutrients resulted in transient and relatively minor disruptions of plankton community structure. The study emphasizes two important points as they relate to risk assessment of this chemical. First, there is a greater need to incorporate field testing as an integral component of a tiered risk assessment process. Despite the identification of longer-term, indirect impacts on the zooplankton community, it would appear that the regulated use of this glyphosate-based herbicide, which prohibits direct application to wetlands such as those used in this study, is unlikely to result in the serious impairment of wetland plankton communities, as might have been predicted from the findings of laboratory-based studies of similar glyphosate-based herbicides. Secondly, the findings of significant effects only in the treatment containing both the herbicide and fertilizers implies that effective ecotoxicological risk assessments should also consider scenarios in which other contaminants or stressors may co-occur in the receiving system, as the possibility exists for joint activity. Addressing the significance of complex ecosystem-level responses to complex mixtures of contaminants, as was done in this study, will contribute to more ecologicallyrelevant ecotoxicological risk assessments.

Assessment and conclusion by applicant:

Not relevant by title/abstract: Non-EU monitoring study. The tested formulation is not the representative formulation for the glyphosate EU renewal, thus article is not relevant.

Further points of clarification:

The representative formulation is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Replicated split-wetland experiment was conducted to investigate the effects of a nominal concentration of 2.88 mg acid equivalents/L of the glyphosate herbicide Roundup WeatherMax, alone or in combination with nutrient additions, on the changes in the phytoplankton and zooplankton communities.

The experimental design and the analytical verification were described in detail in Baker et al. (2014) (also assessed by RMS). The full experimental design here in consisted of 3 "Glyphosate" wetland halves and 3 "Glyphosate+Nutrients" wetland halves, each with a paired untreated half (12 experimental units in total, from six bisected wetlands).

A worst case contamination of wetlands with the herbicide Roundup WeatherMax in combination with fertilizer nutrients resulted in transient and relatively minor disruptions of plankton community structure.
Significant effects were only found in the treatment containing both the herbicide and fertilizers.
The co-application of herbicide and nutrients resulted in an immediate but transient decline in dietary quality of phytoplankton (8.3 % decline in edible carbon content/L) and zooplankton community similarity (27 % decline in similarity and loss of three taxa).
These effects were not evident in wetlands treated only with the herbicide (only a small but significant decline in zooplankton richness of 1.7 ± 0.55 taxa ($p = 0.019$, $n = 3$, $\alpha = 0.212$, paired one-tailed t-test), with no attendant declines in zooplankton abundance or community similarity, nor in phytoplankton quality or amounts).
Unlike after the first application, one day after the second application of herbicide, there were no significant changes observed in phytoplankton or zooplankton endpoints in the herbicide and nutrient treated wetlands. For the herbicide only treated wetlands, phytoplankton abundance and quality appeared to decline, but not significantly. However, the richness of zooplankton in the herbicide alone treated wetlands was reduced by an average of 2.7 ± 0.6 taxa compared to controls ($p = 0.008$, $n = 3$, $\alpha = 0.212$, one-tailed paired t-test). Zooplankton abundance and community similarity were not significantly different between treatment and control halves as a result of the second herbicide application for either treatment.

Indirect effects of the herbicide-nutrient mixture were evident in mid-summer, when glyphosate residues were no longer detectable in surface water. Zooplankton abundance tripled, and zooplankton taxa richness increased by an average of four taxa in the herbicide and nutrient treated wetlands.

Increased abundance of plankton was unlikely to have been a direct stimulatory effect of the herbicide, but it may have been a result of the combination of indirect (from the first application of herbicide) and direct (from the second application of herbicide) effects. The loss of some zooplankton taxa during the initial herbicide application might have released the remaining zooplankton and phytoplankton assemblages from competition and predation. It is also hypothesized that this observation represents an indirect effect of the reduction of zooplankton grazing pressure on some phytoplankton taxa in the community, resulting from the significant loss of zooplankton abundance after the first herbicide application. The timing of the increasing abundance and richness of zooplankton occurred approximately in parallel to the reduction of emergent vegetation; when the treated sides of all wetlands had visibly reduced macrophyte cover (average of 19 % reduction; see Baker et al. 2014).

Based on the above, RMS considers that this study may be relevant to address indirect effects issues.

RMS however notes that significant reduction in plant cover on treated sides of wetlands relative to their control sides following glyphosate herbicide application is not surprising, as reducing plant cover is the intended purpose of glyphosate herbicides (information obtained from Mudge, 2019, also assessed by RMS). The purpose of the additional glyphosate application directly targeting the macrophyte community was to maximize the possibility of indirect impacts of glyphosate herbicides on the invertebrate or amphibian communities through direct effects to the plant community. However, this consistent amount of herbicide applied directly to the plant community on the treated sides of all wetlands was also much higher than the dose received through the different treatment concentrations applied directly to the water's surface.
This study would be then relevant only for aquatic uses where emergent macrophytes are directly exposed.

The study is of limited relevance as the exposure of emergent macrophytes (directly sprayed) was considerably higher than expected from a contamination via run-off/drift and may have resulted in indirect effect on phytoplankton and zooplankton communities.

RMS notes that measured concentrations of glyphosate was far below the nominal targeted (2.88 mg acid equivalents/L) and fastly decreased in the wetlands. Mean of 799 ±644 µg a.e./L (n=6, i.e. including both "glyphosate" and "glyphosate+nutrients") was found at 1 day post-application, 108 ± 112 µg a.e./L at 3 days post-application and 13 ± 11 µg a.e./L at 7 days post application. Despite having the same target glyphosate concentrations, glyphosate residues appeared (not statistically significant) to decline more slowly in the herbicide with nutrients-treated wetlands than the herbicide alone-treated wetlands, where the herbicide and nutrient wetlands had higher glyphosate concentrations of 1173.1 ± 1256.5 µg a.e./L on Day 1 ($p = 0.398$, $n = 3,3$, $\alpha = 0.244$, paired two-tailed t-test) and 195.1 ± 205.1 µg a.e./L on Day 3 ($p = 0.239$, $n = 3,3$, $\alpha = 0.244$, paired two-tailed t-test) than was observed in the herbicide alone-treated wetlands had glyphosate concentrations of 424.5 ± 343 µg a.e./L on Day 1 and 22.3 ± 18.3 µg a.e./L on Day 3.
This high variability could not be explained by any difference of water volume or microbial respiration rates. RMS considers the study reliable with restrictions.

The study authors also suggest that ecotoxicological risk assessments should also consider scenarios in which other contaminants or stressors may co occur in the receiving system, as the possibility exists for joint activity.

For regulatory risk assessment, the study is of limited relevance as the exposure of emergent macrophytes (directly sprayed) was considerably higher than expected from a contamination via run-off/drift.

For assessment of indirect effects, the study can be considered relevant and reliable with restriction.

Data point:	CA 9
Report author	Xu Yanggui *et al.*
Report year	2017
Report title	Effects of glyphosate based herbicides on survival, development and growth of invasive snail (Pomacea canaliculata)
Document No	Aquatic Toxicology, (2017) Vol. 193, pp. 136 143
Guidelines followed in study	None

Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: **as provided in the AIR5 dossier (KCA 9) as proposed by applicant:** **See RMS analysis in RMS comment box**	Not relevant by title/abstract

Full summary of the study according to OECD format

The results showed that glyphosate induced acute toxicity to the snail only at high concentrations (96 h LC50 at 175 mg/L) unlikely to occur in the environment. Long term exposures to glyphosate at sublethal levels (20 and 120 mg/L) caused inhibition of food intake, limitation of growth performance and alterations in metabolic profiles of the snail. It is worth noting that glyphosate at 2 mg/L benefited growth performance in P. canaliculata. Chronic exposures of glyphosate significantly enhanced overall metabolic rate and altered catabolism from protein to carbohydrate/ lipid mode. Cellular responses in enzyme activities showed that the exposed snails could increase tolerance by their defence system against glyphosate induced oxidative stress, and adjustment of metabolism to mitigate energy crisis. The study displayed that sublethal concentrations of glyphosate might be helpful in control of the invasive species by food intake, growth performance and metabolic interruption; whether environmental relevance of glyphosate (≤2 mg/L) benefits population growth of P. canaliculata is still inconclusive, which requires further field study.

Materials and methods

Chemicals and reagents
Chemical standard for glyphosate (C3H8NO5P) was purchased from Dr. Ehrenstorfer (98%, Germany). The glyphosate stock solution of 1000 mg/L was prepared with Milli-Q water of 18.2 MΩ cm (Millipore, Billerica, MA, USA) and stored in the dark at 4 °C. Reagents for toxicity bioassay were prepared for reconstituted moderately hard water (MHW) using the ISO 6341 recipe (ISO, 2012): 294.0 mg/L calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$, 96.0%, China), 123.0 mg/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, 99.0%, China), 64.8 mg/L sodium bicarbonate ($NaHCO_3$, 99.5%, China), 5.75 mg/L potassium chloride (KCl, 99.5%, China).

Acute toxicity
Egg clutch of the golden apple snail P. canaliculata, being bright pinkish-red and gelatinous, was collected from an ecological farming system (23°09′N, 113°21′E) of South China Agricultural University in Guangzhou, China. Being transported to the laboratory, egg mass was cultured in an incubator with temperature at 28 ± 2 °C, dissolved oxygen>5 mg/L, humidity of 75%, and light/dark cycle of 14/10 h. Such experiments were performed in compliance with the guidelines from South China Agricultural University. Newly hatched snails (< 24 h) were used for acute toxicity bioassay. 10 concentrations of glyphosate ranged from 2 mg/L to 300 mg/L together with a MHW water control were applied for 96 h exposure. Briefly, 10 newly hatched snails were randomly assigned to 100 mL of target solution in a 150-mL beaker. There were three replicates for each treatment. Incubation condition was under temperature of 28 ± 2 °C, humidity of 75%, and light/dark cycle of 14/10 h. At the end of 24, 48, 72 and 96 h exposure, dead animals were recorded and removed from the beaker.

Chronic toxicity
Based on data of acute toxicity, environmental relevant and sublethal levels of glyphosate were used for the following chronic tests.

Hatching rate
This part of work aimed to check influence of glyphosate spraying during agricultural activity on hatching of the golden apple snail eggs. By simulation, individual egg was soaked for 10 s in glyphosate solutions at environmental levels of 0.02, 0.2 and 2 mg/L, sublethal dose of 20 mg/L as well as the MHW control. Afterwards, the exposed eggs were placed in 96-well plate with the MHW for three weeks. There were three replicates with sample size of 40 snails in each treatment. Incubation condition was the same as that for egg culture. On days 7, 14 and 21, the number of hatching was recorded.

Physiological responses
Snails within 7-day post hatched in similar size (length: 5.5 ± 0.16 mm; weight: 0.05 ± 0.003 g (mean ± standard deviation)) were used in this part. The exposure was conducted at 28 ± 2 °C in an incubator with humidity of 75% and light/dark cycle at 14/10 h. There were four glyphosate treatments of 0.2, 2, 20 and 120 mg/L together with a MHW control. For each treatment, three replicate glass tanks (40×30×20 cm) were applied and each tank initially had 10 hatched snails. Plastic netting was placed on the surface of solution to keep animals in water. Snail density was kept at one snail per 50 mL solution during the whole test. Snails were fed ad libitum daily on a diet of fresh lettuce after being rinsed by Milli-Q water, Kimwipes and airdried for 1 h. On the days of evaluating food intake of the snails, lettuce was weighted before feeding; after 24 h, the residue of lettuce in the tank was collected, rinsed by Milli-Q water, Kimwipes and air-dry for 1 h and weighted. Mortality was recorded and dead individuals were removed daily to keep water quality. Each tank had water change once every week and water volume was adjusted to match with the number of snails left in the tank. The test lasted for 135 days. On days 15, 45, 75, 105 and 135, food intake and growth performance of length and weight were studied. These parameters were calculated using the following equations:

Food intake (g/snail/d) = $(\Delta F_t/No_{snail})$,
Length change (%) = $(\Delta_L/L_0) \times 100\%$,
Weight change (%) = $(\Delta W/W_0) \times 100\%$,

where ΔF_t is the mean of food intake (g) on day *t*, and No_{snail} is the number of exposed snails; ΔL is the mean difference in length (mm) between Day t and Day 0, and L_0 is the initial mean length measured on day 0; ΔW is the mean difference in wet weight (g) between Day t and Day 0, and W_0 is the initial mean weight measured on day 0. On days 45, 75 and 105, metabolic responses of the exposed snails were evaluated by oxygen consumption (*OC*) and ammonia excretion (*AE*) by using bottle-water method. In brief, five individuals within each replicate were taken from the same replicate of the exposure tanks, and then placed into the respiration chamber containing the same exact concentration level. After 24 h incubation in the chamber, no mortality was observed. Dissolved oxygen (*DO*) in each chamber was measured at the start and after 24 h by the Portable Dissolved Oxygen Instrument (YSI 58) while the amount of ammonia produced in 24 h was determined using standard indophenol blue method. One chamber without snail was run as control in parallel. Each treatment and control had three replicates. The *OC* and *AE* were estimated by the following equations:

OC (mg O_2/g body weight/h) = $[(DO_t - DO_0)_{snail} - (DO_t - DO_0)_{control}] \times V/(W \times t)$
AE (mg N/g body weight/h) = $[(N_t - N_0)_{snail} - (N_t - N_0)_{control}] \times V/(W \times t)$

Where DO_t and DO_0 are the dissolved oxygen at the time t and 0 in the respiration chamber with/without snail (mg/L), V is the solution volume in the respiration chamber (L), W is the combined wet weight for all snails in the replicate (g), and t is the experimental time (h); Nt and N0 are the ammonia concentration at the time t and 0 in the respiration chamber with/without snail (mg/L).
The O:N atomic ratio, namely the atoms of oxygen consumed per atom of N excreted, was applied to evaluate the proportion of protein related to carbohydrates/lipids for catabolism:

O:N = (*OC*/16)/(*AE*/17)

Enzyme assays
Not reported by RMS (see RMS comment below)

Results

Acute toxicity of glyphosate

The results from acute toxicity assay showed that 96-h LC50 of glyphosate to the newly-hatched golden apple snails was 175 mg/L (Table 1).

Table 1 Lethal concentration 50 (LC50) and 95% confidence interval (n =3) of glyphosate on the golden apple snail Pomacea canaliculate (< 24 h).

Exposure time (h)	LC50 (mg/L)	95% confidence interval (mg/L)
24	178.2	176.4–180.0
48	176.5	175.4–177.5
72	176.2	175.3–177.1
96	175.1	174.7–175.6

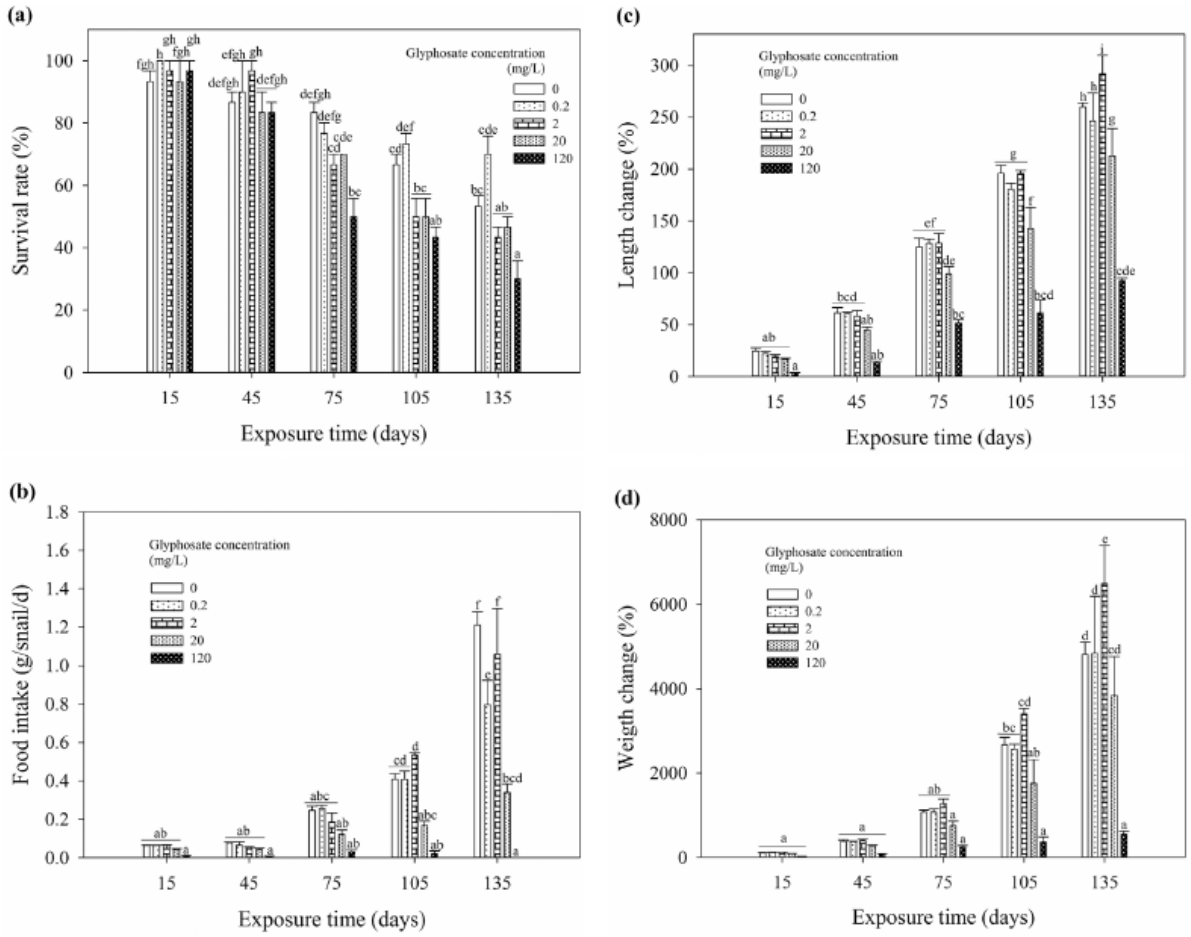
Chronic toxicity of glyphosate on the snail development

Glyphosate spraying test indicated that there was no statistically difference in hatching rate of the golden apple snail eggs among glyphosate treatments within 21 days of exposure (Fig. A1 in Supplementary material). Laboratory control of hatching success in P. canaliculata was only up to an average of 40% while Barnes et al. (2008) also reported that hatching efficiency of the invasive species P. insularum in the laboratory was as low as 30% with high variations (7–90%). Comparably, zebrafish embryo Danio rerio acutely exposed to sublethal concentration of glyphosate (50 mg/L) surrendered to cardiovascular toxicity in structural abnormalities, irregular heart looping and decreased heartbeats (Roy et al., 2016). Thus, hatching test in the golden apple snail P. canaliculata might not be a qualified biomarker in the laboratory.

Significant difference in survival rate of the snails was found during 135-day exposure to glyphosate (one-way ANOVA: $F_{24,50}$ =20.706, $p < 0.05$; Fig. 1a). Relative to the MHW control, the treatment at 120 mg/L of glyphosate demonstrated a significant difference on/after 75-day exposure (Fig. 1a). It is worth noting that survival rate displayed no difference between glyphosate exposures at environmental relevance of 2 mg/L and sublethal levels of 20 or 120 mg/L. Survival dropped off considerably in the 2, 20 and 120 mg/L treatments compared to the 0.2 mg/L treatment on/after 105 days. However, no any difference was detected between the control and any of the lower three concentrations (e.g. 0.2, 2 and 20 mg/L) at any timepoint.

Physiological responses of snail in food intake (one-way ANOVA: $F_{24,47} = 29.156$, $p < 0.05$; Fig. 1b), length change (one-way ANOVA: $F_{24,50} = 66.538$, $p < 0.05$; Fig. 1c) and weight change (one-way ANOVA: $F_{24,50}$ =20.939, $p < 0.05$; Fig. 1d) also showed significant differences in a glyphosate concentration-dependent manner. The three parameters shared similar patterns that (a) glyphosate treatment at 120 mg/L presented remarkable inhibition effects in amount of food intake and growth performances on/after 105-day exposure while glyphosate at 20 mg/L presented the same influence in some cases; (b) no difference was detected between the control and glyphosate treatment at 0.2 mg/L except food intake on day 135; (c) after exposure of 135-day, the glyphosate at 2 mg/L could significantly benefit length/ weight gain. Firstly, glyphosate induced reduction of food intake at relative high doses (Fig. 1b) is possibly a key factor to limit growth performance (Fig. 1c, d), and even survival success (Fig. 1a).

Fig. 1. Physiological responses of the golden apple snail Pomacea canaliculate (n = 3) in (a) survival rate, (b) food intake, (c) length change, and (d) weight change exposed to various concentrations of glyphosate. Bars with different letters denote significantly different means ($p < 0.05$, Student-Newman-Keuls test).

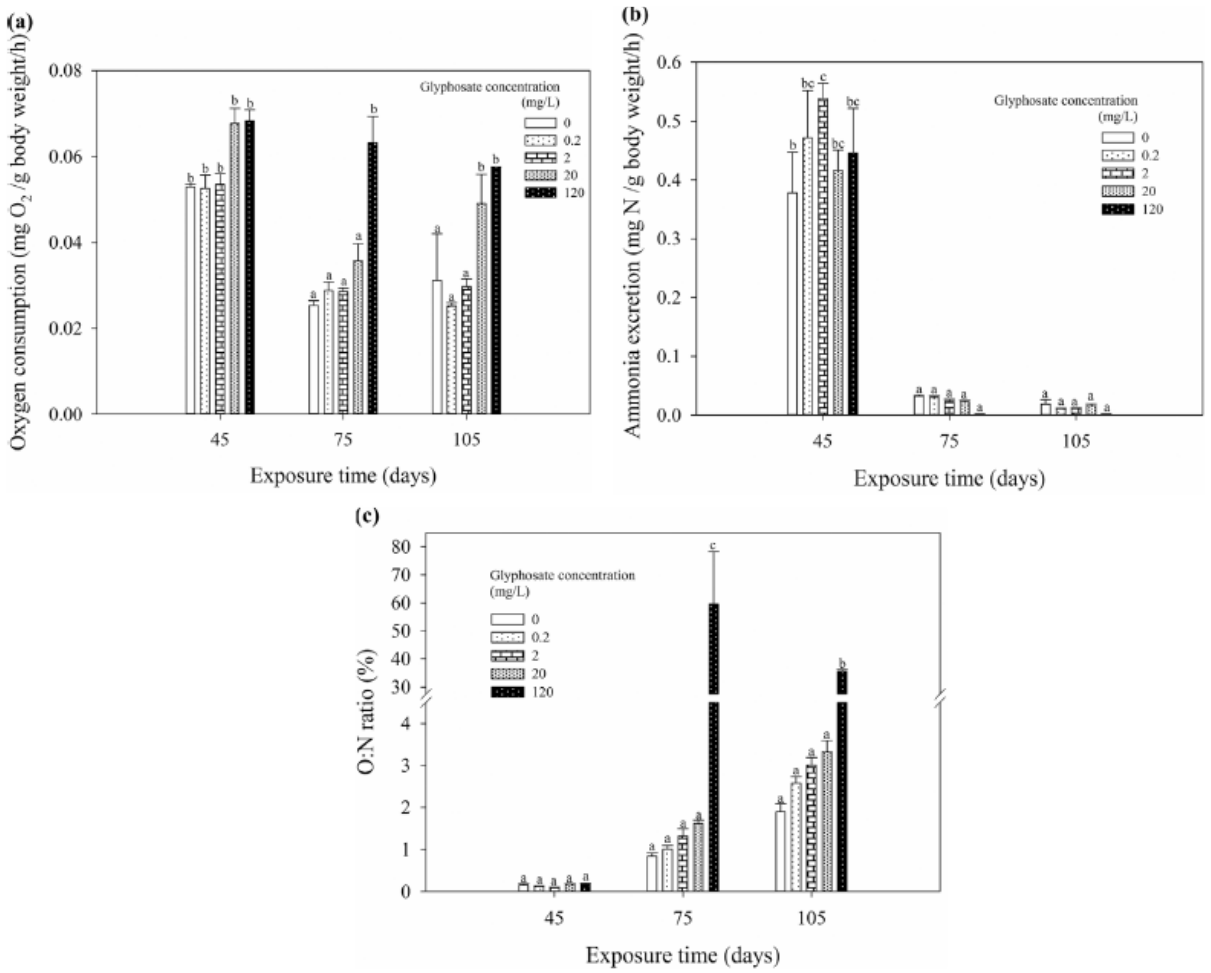


Chronic toxicity of glyphosate on metabolic rates of the snail

For oxygen consumption, the control and two lower doses of glyphosate at 0.2 and 2 mg/L decreased at greater size of the snails, the 20 mg/L dose resulted in higher oxygen consumption on 105-day close to that on 45- day after initially dropping on 75-day, and the highest dose at 120 mg/ L glyphosate appeared to remain it relatively constant (Fig. 2a). The larger size of the snails appeared to have decreased ammonia excretion on 75-day and thereafter (Fig. 2b). Additionally, the two highest concentrations resulted in greater oxygen consumption, except for the 20 mg/L treatment on day 75. This is probably due to an increase in energy demand under glyphosate stress because chemical detoxification is an energy demanding process (Rissoli et al., 2016). In turn, such increased metabolism probably encourages the uptake and accumulation of chemical into the body via higher respiration in aqueous solution, leading to intensified toxic effect to the species (Li, 2014).

It is generally accepted that low O:N ratios relate to metabolism where protein is heavily used, while high values correspond to depletion of carbohydrate/lipid reserves (Xiao et al., 2014). The data from this study showed that the golden apple snails primarily used protein catabolism at ≤ 20 mg/L of glyphosate treatments; after 75-day exposure, glyphosate at only 120 mg/L significantly altered catabolism of the species to carbohydrate/lipid mode (Fig. 2c).

Fig. 2. Metabolism and catabolism of the golden apple snail Pomacea canaliculate (n = 3) in (a) oxygen consumption, (b) ammonia excretion, and (c) O:N ratio exposed to various concentrations of glyphosate. Bars with different letters denote significantly different means ($p < 0.05$, Student-Newman-Keuls test).



Chronic toxicity of glyphosate on enzyme activities of the snail
Not reported by RMS (see RMS comment below)

Conclusion

In the present work, effects of glyphosate on survival, development and growth of the invasive snail P. canaliculata were studied. The data suggests that glyphosate can cause acute toxicity to the snail but only at high concentrations unlikely to occur in the natural environment; long term sublethal exposures (e.g. 20 and 120 mg/L) to glyphosate reduced uptake of food, limited growth performance and altered metabolic profiles of the snails. Glyphosate, being a chemical stressor (FBPase, ACP and ALP), would enhance oxygen consumption and alter catabolism sources in the snail (O:N ratio and G6PDH). Simultaneously, the golden apple snails enhanced their tolerance for adaptation to glyphosate load by defense system (G6PDH) against oxidative stress (SOD and CAT), and metabolic shift (LDH and MDH) for increased energy demand (ATPase). It is worth noting that glyphosate at environmentally relevant concentration of 2 mg/L benefited length/weight gain of the snail; however, further field study is required to verify whether environmental levels of glyphosate is one of factors contributing successful colonization of the golden apple snails in Asia. Nevertheless, this study has limitations. Statistical analysis in enzyme activities is not robust due to limited alive snails left after 135-day exposure to glyphosate. More biological replicates would help to detect significant difference but long-term exposures during biologically chronic test is always challenged by confounding factors irrespective of time and effort consumptions. Field work is lacking in this study to double confirm glyphosate influence to the golden apple snails. As this is a fundamental study in potential effects of practical glyphosate application on survival, development and growth in the invasive species of golden apple snails, further research is warranted.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by title/abstract: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). Enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological regulatory risk assessment.

Assessment and conclusion by RMS:

In this study it was investigated whether application of glyphosate would benefit population growth of an alien invasive species, the golden apple snail Pomacea canaliculata in China.
Snails were kept in the water.
The report comprises three parts:
- in the first part an acute toxicity was conducted (96h);
- in the second part a chronic assay lasted for 135 days to glyphosate exposures was taken with a focus on physiological response (food intake, growth performance of length and weight) and metabolic rate of the snail (oxygen consumption, ammonia excretion);
- in the third part of the study, 11 enzymes were adopted to study cellular response of the golden apple snail against glyphosate after chronic exposure. (superoxide dismutase (SOD) and catalase (CAT) (oxidative stress), lactate dehydrogenase (LDH; glycolysis), malate dehydrogenase (MDH; citric acid cycle), fructose-1,6-bisphosphatase (FBPase; gluconeogenesis), glucose-6-phosphate dehydrogenase (G6PDH; pentose phosphate pathway), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (protein metabolism), acid phosphatase (ACP) and alkaline phosphatase (ALP) (phosphate metabolism), and adenosine triphosphatase (ATPase; energy metabolism)). The study authors that only tendency instead of significant difference among treatments were shown and that statistical analysis in enzyme activities is not robust due to limited alive snails left after 135-day exposure to glyphosate. These were therefore not considered thereafter by RMS.

Pure glyphosate was used.
All these parameters are deemed relevant by RMS.

Results:
An endpoint for mortality was set : 96h LC50 = 174.7 mg/L (95% CI: 174.7-175.6)
Long-term exposures to glyphosate at 20 and 120 mg/L caused inhibition of food intake, limitation of growth performance and alterations in metabolic profiles of the snail. Glyphosate at 2 mg/L benefited growth performance in P. canaliculata.
No analytical verification was conducted.
Results on enzyme activities are considered not reliable (and therefore not reported in the summary above).

The study is considered relevant and reliable with restrictions (except enzyme activities).

B.9.2.8.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 8.2.6
Report author	Lam C. H. et al.
Report year	2020

Report title	Toxicity of herbicides to cyanobacteria and phytoplankton species of the San Francisco Estuary and Sacramento-San Joaquin River Delta, California, USA.
Document Source	Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering (2020), Vol. 5, pp. 107-118
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Three species of microalgae (*Microcystis aeruginosa*, *Chlamydomonas debaryana*, and *Thalassiosira pseudonana*) were exposed to glyphosate-based herbicide at nominal concentrations of 0, 0.7, 7 and 70 mg glyphosate/L in 96-well plates for 5-8 days. The experiment was performed with three wells per replicate, five replicates per treatment, for a total of 15 wells.
Glyphosate-based herbicide inhibited algal growth of the three species tested only at the highest concentrations tested, which was 4.9 x 10^4 µg/L for *M. aeruginosa*, 7.0 x 10^4 µg/L for *T. pseudonana* and 7.0 x 10^4 µg/L *C. debaryana*, which was less sensitive to glyphosate than *M. aeruginosa* and *T. pseudonana* and growth was significantly, but not completely inhibited.

Materials and methods

Three species of microalgae, *M. aeruginosa* (cyanobacteria), *C. debaryana* (green algae), and *T. pseudonana* (diatom) found in the San Francisco Estuary (SFE) / Sacramento-San Joaquin River Delta (Delta) were used. The cultures of *M. aeruginosa* and *C. debaryana* were established by single cell isolation from algal samples collected from the Delta during October 2016 and September 2017. Local isolates of *M. aeruginosa* and *C. debaryana* were used in order to eliminate the problem of adaptation from other geographical locations. Briefly, single algal cells of interest were picked up using glass micropipettes under a microscope, rinsed in small volumes of sterile media several times (ca. 100 µL), and then transferred into 96-well plates. After single cell isolation, *M. aeruginosa* and *C. debaryana* were routinely maintained in 100 mL of CB media at 25 °C with continuous shaking on orbital shakers and weekly media changes. *T. pseudonana* was purchased from the Culture Collection of Algae at the University of Texas. *T. pseudonana* was cultured in F/2 media at 18 °C. Salinity was adjusted to 8 ppt with sea salt (Instant Ocean Spectrum Brands, Blacksburg, VA, USA). All cultures were kept in environmental controller chambers (Percival Scientific, Inc. Perry, IA, USA) that provided cool white fluorescent lighting at 16/8 hour light/dark cycles.

Preparation of experimental solutions and chemical analysis
The commercial formulation of glyphosate (Roundup Custom®) was obtained from Helena Agri-enterprises, LCC. Experimental solution was prepared by spiking the chemical into CB media for *M. aeruginosa* and *C. debaryana*, and by spiking into F/2 media (salinity adjusted to 8 ppt) for *T. pseudonana* and based on active ingredient concentrations on the manufacturer's label. Small aliquots of the experimental solutions were saved for chemical analysis. The concentrations of glyphosate in the experimental solutions were measured by ELISA (Abraxis LLC, Warminster, PA, USA). The nominal (applied) and measured concentrations used for each species are listed in Table 1.

Table 1. The nominal and measured concentrations of glyphosate used.

Chemical	Species	Nominal concentration (mg L^{-1})	Measured concentration (mg L^{-1})
Glyphosate	*Chlamydomonas*	0	Below limit of detection
		0.7	0.69 ± 0.02
		7	7.54 ± 0.38
		70	76.9 ± 0.03
	Microcystis	0	Below limit of detection
		0.7	0.71 ± 0.01
		7	7.39 ± 0.07
		70	49.1 ± 0.87
	T. pseudonana	0	Below limit of detection
		0.7	0.65 ± 0.01
		7	6.50 ± 0.05
		70	68.4 ± 0.54

Evaluation of growth inhibition

Exposure bioassays were run in 96-well plates. Algal cells in the exponential growth phase (3–5 days old) were spiked into the experimental solutions to obtain an initial cell density of 10,000 cells/mL. 340 µL of the experimental solutions with cells were placed into each 350 µL test well. The experiment was performed with three wells per replicate, five replicates per treatment, for a total of 15 wells.

For each treatment, a well containing only the experimental solution was included as a negative control. Absorbance of each well was measured daily for eight days at 750 nm (for turbidity) using an Infinite M200 plate reader (TECAN, Männedorf, Switzerland). At the same time, absorbance at 684 nm (*M. aeruginosa* and *C. debaryana*) and 676 nm (*T. pseudonana*) were measured daily. Plate position in the environmental controller chambers were randomized daily.

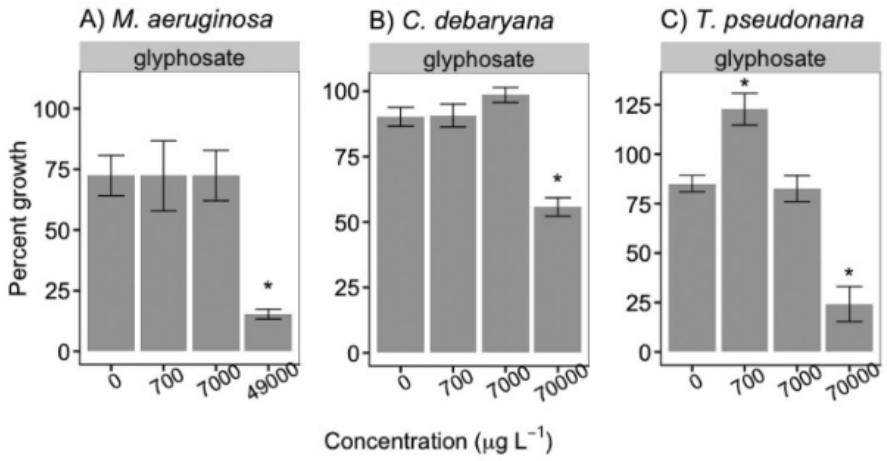
Significant differences between the absorbance at the exponential growth phase (Day 5-8) of each concentration and of the control were compared for each species using one-way ANOVA. A post-hoc Dunnett's test was performed following a significant ANOVA.

Results

Bioassays

Glyphosate affected growth for all three species (*M. aeruginosa*: P = 0.0009; *C. debaryana*: P < 0.0001; *T. pseudonana*: P <0.0001) (Fig. 1). Glyphosate inhibited algal growth only at the highest concentrations tested, which was 4.9 x 10^4 µg/L for *M. aeruginosa* (P = 0.0018) and 7.0 x 10^4 µg/L for *T. pseudonana* (P = 0.0084). At 700 µg/L, glyphosate significantly enhanced *T. pseudonana* growth by almost 50% over the control (P = 0.0271). *C. debaryana* was less sensitive to glyphosate than *M. aeruginosa* and *T. pseudonana*, and growth was significantly (P < 0.0001), but not completely inhibited at 7.0 x 10^4 µg/L.

Figure 1. Growth inhibition for *Microcystis aeruginosa* (A), *Chylamydomonas debaryana* (B) and *Thalassiosira pseudonana* (C) exposed to glyphosate at different concentrations. Asterisks (*) indicate statistical differences from the control group (P < 0.05).



Conclusion

After 5-8 days of exposure, glyphosate inhibited algal growth of the three species tested only at the highest concentrations tested, which was 4.9 x 10^4 µg/L for *M. aeruginosa* and 7.0 x 10^4 µg/L for *T. pseudonana*. At 700 µg/L, glyphosate-based herbicide significantly enhanced *T. pseudonana* growth by almost 50% over the control. *C. debaryana* was less sensitive to the glyphosate-based herbicide than *M. aeruginosa* and *T. pseudonana*, and growth was significantly, but not completely inhibited at 7.0 x 10^4 µg/L.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: The composition of the Roundup Custom used in the test cannot be confirmed. Roundup Custom is meant for aquatic uses so would not contain surfactants. It is not clear from the study if the product was tested with an approved surfactant added or not as would be detailed on the label (there is limited information in the paper on the label). However, Roundup Custom is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). In addition, as mentioned above Roundup Custom is meant for aquatic uses and aquatic uses are not on the current GAP table for the glyphosate EU renewal.

Further points of clarification:
The original Roundup formulation contains POEA surfactant which is not permitted in formulated herbicidal products in the EU. The surfactant system in the formulated product used in this study, was not confirmed by the authors. Therefore, the findings of this paper should be treated with a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The effects of glyphosate, imazamox and fluridone are investigated on natural isolates of phytoplankton and cyanobacteria.
Three species of microalgae found in the San Francisco Estuary (SFE)/Sacramento-San Joaquin River Delta (Delta) (Microcystis aeruginosa, Chlamydomonas debaryana, and Thalassiosira pseudonana) were exposed at a range of concentrations (0, 0.7, 7 and 70 mg glyphosate/L) for 5–8 days. The test item is Roundup Custom (meant for aquatic uses). No sufficient information in the study report is available to demonstrate whether the tested formulation is comparable with the representative formulation. The sudy is considered less relevant but supplementary due to formulation issue.
Glyphosate inhibited algal growth only at the highest concentrations tested, which was 4.9 x 10^4 µg /L for M. aeruginosa and 7.0 x 10^4 µg /L for T. pseudonana (NOEC = 7 mg/L for both species). At 700 µg /L, glyphosate significantly enhanced T. pseudonana growth by almost 50% over the control (hormetic effect is hypothetised by authors). Authors stated that fluridone was the only herbicide that inhibited the microalgae at environmentally relevant concentrations in this study and susceptibility to the herbicide depended on the species. Analytical verifications have been made. Only graphics are available (no biological data reported).
RMS notes that only NOEC were available, no EC50 was calculated that could be used in the risk assessment. RMS considers these tested concentrations as very worst-case (Roundup Custom is meant for aquatic uses) but these results are nevertheless informative.
The study is considered less relevant but supplementary (different formulation was tested) and reliable with restrictions.

Data point:	CA 9
Report author	Omran N. E. *et al.*
Report year	2016
Report title	The endocrine disruptor effect of the herbicides atrazine and glyphosate on *Biomphalaria alexandrina* snails.
Document No	Toxicology and industrial health (2016), Vol. 32, No. 4, pp. 656-665
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

Atrazine (AZ) and glyphosate (GL) are herbicides that are widely applied to cereal crops in Egypt. The present study was designed to investigate the response of the snail *Biomphalaria alexandrina* (Mollusca: Gastropoda) as a bioindicator for endocrine disrupters in terms of steroid levels (testosterone (T) and 17β-estradiol (E)), alteration of microsomal CYP4501B1-like immunoreactivity, total protein (TP) level, and gonadal structure after exposure to sublethal concentrations of AZ or GL for 3 weeks. In order to study the ability of the snails' recuperation, the exposed snails were subjected to a recovery period for 2 weeks. The results showed that the level of T, E, and TP contents were significantly decreased ($p \leq 0.05$) in both AZ- and GL-exposed groups compared with control (unexposed) group. The level of microsomal CYP4501B1-like immunoreactivity increased significantly ($p \leq 0.05$) in GL- and AZ-exposed snails and reach nearly a 50% increase in AZ-exposed group. Histological investigation of the ovotestis showed that AZ and GL caused degenerative changes including azoospermia and oocytes deformation. Interestingly, all the recovered groups did not return back to their normal state. It can be concluded that both herbicides are endocrine disrupters and cause cellular toxicity indicated by the decrease of protein content and the increase in CYP4501B1-like immunoreactivity. This toxicity is irreversible and the snail is not able to recover its normal state. The fluctuation of CYP4501B1 suggests that this vertebrate-like enzyme may be functional also in the snail and may be used as a biomarker for insecticide toxicity.

Materials and methods

Herbicides
Glyphosate: a product with commercial name ''Herfosate'' in Egypt was used in this study.
Percentage of isopropylamine equals 48% w/v, and inert ingredients equal 52% w/v. One liter of Herfosate equals 480 g of active ingredient (480 g/L). The stock solution was prepared by adding 2.1 ml (= 1 g) of Herfosate and was completed to 1000 ml by adding dechlorinated tap water.
Atrazine: a product with commercial name Atrazex in Egypt was used in this study.
The stock solution was prepared by adding 1 g of atrazine powder to 1000 ml dechlorinated tap water.
Experimental design
Adult snails of *B. alexandrina* with shell diameter ranging between 8 and 10 mm were purchased from the snail control unit, the Theodor Bilharz Research Institute (TBRI; Egypt). They were kept in glass containers in dechlorinated tap water under constant aeration at least for a period of 4 weeks to be

acclimatized with the laboratory conditions (pH = 7.4 ± 0.2 and temperature of 27 ± 2°C). They were fed daily with fresh lettuce, and water was renewed weekly.
The efficiency of the two herbicides against adult snails was determined according to WHO (1965). A series of concentrations was prepared from the stock solution (1000 ppm) of each investigated pesticide to determine the lethal concentration at 50% and 10% (LC50 and LC10). Exposure and recovery periods were 24 h each. Mortality rates were recorded and data analysis aiming to deter- mine LC_{50} and LC_{10} was carried out using a Finny program with reliability interval of 95%.
A total of 250 adult snails (8-10 mm) were divided into 5 groups (50 snails each) as follows: Control group: received no treatment,
AZ-treated snails: treated for 3 weeks with LC_{10} AZ (Az),
GL-treated snails: treated for 3 weeks with, LC_{10} GL (GL),
AZ-recovered snails (AzR): these are snails treated with LC_{10} Az for 3 weeks then replaced into a dechlorinated water for a recovery period of 2 weeks,
and GL-recovered snails (GlR): these are snails treated with LC_{10} GL for 3 weeks then replaced into a dechlorinated water for a recovery period of 2 weeks.
Herbicide solutions were changed every 24 h with new prepared ones to avoid the effect of storage. All snails fed boiled lettuce and kept in glass aquaria. At the end of the exposure period, the snails were dissected and their gonads were stored in -80°C until use for steroid and cytochrome analysis.

Steroid extraction
Total T and total E were extracted as described in Janer et al. (2005) with some modifications. Briefly, 0.1 g wet weight of gonad tissue was homogenized in ethanol, and frozen overnight at -80 °C. Homogenate was then extracted with ethylacetate twice. The organic extract was evaporated under nitrogen, and resuspended in 1 mL methanol containing 0.1% potassium hydroxide, and kept at 45 °C for 3 h. After the saponification step, double distilled water (2 mL) was added, and the sample was extracted with dichloromethane (3 x 3 mL^2).

Steroid analysis
Hormone concentrations (T and E) were assayed according to the manufacture instructions of T EIA kit (Enzo Life Science, Michigan, USA, ADI-900-065) and E EIA kit (Cayman Chemical Company, Michigan, USA, item no. 582251). The limits of the detection were 5.67 pg/mL for T and 19 pg/mL for E. Intraassay coefficients of variation were of 9.5% (T) and 15.8% (E). Interassay coefficients of variation were 11.7% (T) and 6.2% (E).

Subcellular fractionation
Digestive gland gonad complex tissue was homogenized in ice-cold 100 mM monopotassium phosphate (KH_2PO_4)/ dipotassium phosphate (K_2HPO_4) buffer pH 7.4 containing 0.15 M potassium chloride (KCl). Homogenate was centrifuged at 500g for 15 min, the fatty layer removed and the supernatant was centrifuged at 12,000g for 45 min. After centrifugation at 100,000g for 60 min, the supernatant, termed cytosol, was collected and discarded and the pellet (microsome) was resuspended with the same buffer and centrifuged again at 100,000g for 60 min. Microsomal pellets were resuspended in a small volume of 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 containing 0.15 M KCl and 20% (w/v) glycerol.
Total protein
Microsomal protein concentrations were determined using a commercial kit (Biomed Diagnostics, 30175 Hannover, Germany) using bovine serum albumin as a standard.

Determination of CYP4501B1
This enzyme was assayed by enzyme-linked immuno- sorbent assay using a commercial kit. Detection range was 0.156-10 ng/mL and sensitivity was 0.063 ng/mL.

Histological examination of the ovotestis
The hermaphrodite gland (ovotestis) was dissected out of the snails (n = 5 per group) and fixed in 10% formalin for 24 h, dehydrated in a graded series of ethanol and embedded in paraffin wax. Serial tissue sections were cut at 6 mm and stained with hematoxylin and eosin (H&E) stains. The specimens were examined microscopically to assess the gonad development (Olympus microscope CX31; Tokyo, Japan) equipped with an image analyzing system.

Data analysis
The obtained results were statistically analyzed using Student's t test to determine the significant differences between exposed, recovered, and control specimens. The software Statistical Package for Social Sciences version 17.0 for Windows (SPSS; Chicago, Illinois, USA) was used for the analysis. Significance was set at $p < 0.05$.

Results

Only the results for Glyphosate (GL) are presented here.

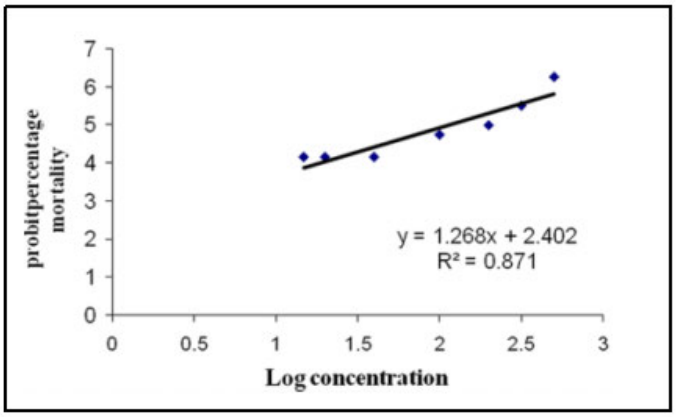
Molluscicidal activity
The molluscicidal activity of GL on *B. alexandrina* snails after 24 h of exposure under the laboratory conditions was determined and is listed in Table 1 and included in Fig. 1. The data obtained indicated that the recorded LC_{50} value was 41.6 ppm for GL. So, the sublethal dose (LC_{10}) was 4.2 for GL.

Table 1. Molluscicidal potency of glyphosate against *Biomphalaria alexandrina* snails (24 hours exposure).

Herbicide	LC_{50}	LC_{10}
Glyphosate	41.6 ppm	4.2 ppm

LC_{50}: lethal concentration at 50%; LC_{10}: lethal concentration at 10%.

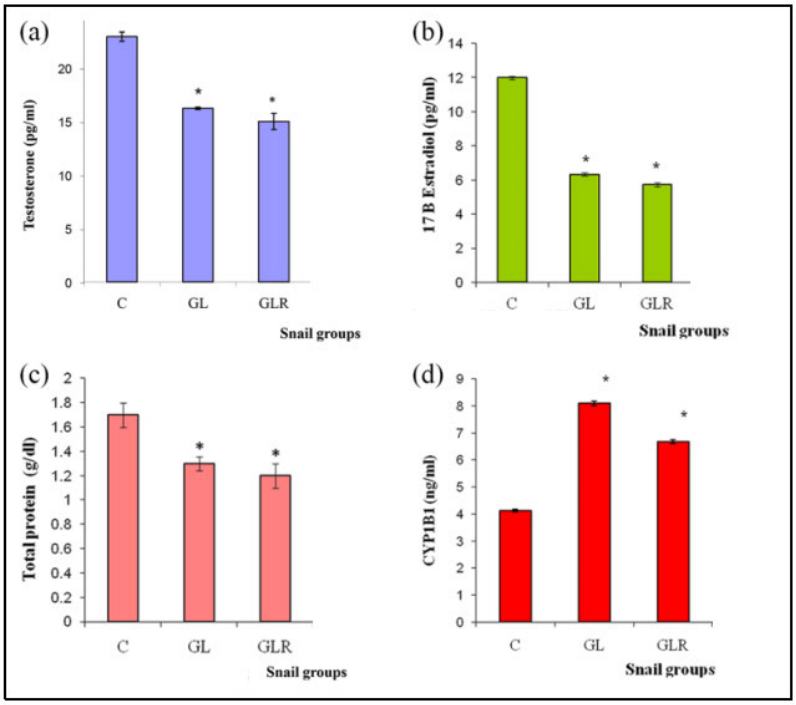
Figure 1. Concentration mortality curve (log vs. probit) of glyphosate against *Biomophalaria alexandrina*.



Steroid level
Total T and E were determined in the gonads of GL-exposed snails after 3 weeks of exposure. Level of T was decreased significantly in GL-exposed snails (18.11 ± 0.13 pg/ml, $p < 0.05$) compared with control (23.03 ± 0.4pg/ml) as indicated in Fig. 2a/2b. The level of E was decreased by nearly 50% in GL- (6.33 ± 0.1 pg/ml) exposed snails compared with control (12.01 ± 0.096 pg/ml). In order to study the ability of the snails' recuperation, snails were transferred into clean fresh water for 2 weeks, and then the levels of T and E were determined. The level of T in the GL (GLR)-recovered groups showed a significant decrease when compared with the control group meaning that the recovered period did not ameliorate its concentration.

The same case was obtained in E level, where GLR showed a significant decrease in E concentration when compared with control group.

Figure 2. Testosterone (a), estradiol (b), total protein and CYP4501B1-like immunoreactivity concentrations (mean + SD) in different snail goups (n = 3/group).*p < 0.05 (t test). C: control; GL: glyphosate-exposed snails; GLR: recovered snails after exposure to glyphosate.



Total protein

TP concentration was significantly decreased in GL-exposed snails compared with the control as indicated in Fig. 2c. On the other side, the recovery period did not ameliorate the protein concentration but in contrast caused a significant decrease in GLR.

CYP4501B1

CYP4501B1-like immunoreactivity of the snail microsomal proteins increased significantly ($p < 0.05$) following their exposure to GL as indicated in Fig. 2d. On the other side, the recovery period lead to a significant decrease of the CYP4501B1-like immuno-reactivity in GLR when compared with GL exposed snails but still high when compared with the control.

Histological examination of the ovotestis

Histological investigation of the ovotestis showed that the control snails, contained mature male and female gametes, spermatozoa and ovum in well-developed acini connected by thin connective tissues. Exposure of the snails to sublethal concentrations of GL for 3 weeks resulted in marked degenerative changes such as lack of most stages of gametogenesis, vacuolation, deformation of oocytes, atretic ova, and infiltration of dark nucleated cell in between acini.

Conclusion

The results obtained showed an inhibition of both E and T in the herbicides-exposed snails indicating the disrupter effect of GL. The present findings showed a significant decrease in the TP (total protein) of the GL-exposed snails which indicate that herbicidal stress might increase the proteolysis activities in the cells or a general disruption in translation. Or it may be due to cell necrosis that indicated by the presence of deformed and atretic oocytes. An increase in CYP4501B1-like immunoreactivity of the snail microsomal proteins following exposure to GL. Histological investigation of GL-exposed snails showed deleterious effect on male and female gametes, absence of almost all the gametogenesis stages,

deformation of oocytes and infiltration of dark nucleated cells between acini. The present study showed that a recovery period did not ameliorate or enhance production of steroid hormones. The toxicity of the investigated herbicides is clear when discussing the inability of the snail to recover the protein levels. The increase in CYP4501B1-like immunoreactivity may indicate the increase in the detoxification process taken by the snail to overcome the herbicide toxicity. This detoxification process then tends to slow down by the recovery period. The fluctuation of CYP4501B1 suggests that this vertebrate like enzyme may be functional also in the snail. In conclusion *B. alexandrina* snails can be used as an indicator for screening on endocrine disrupter chemicals as its response is comparable to vertebrates taking into consideration the advantages of selecting a mollusc assays.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Cellular level end-points are not considered relatable to an EU level ecotoxicology regulatory risk assessment. In addition, a glyphosate based herbicide ('Herfosate' containing 480 g of active ingredient) was tested which is not the representative formulation for the glyphosate EU renewal. Therefore, the article and results presented are not relevant to the EU level regulatory risk assessment / glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The study investigates the response of the snail *Biomphalaria alexandrina* (Mollusca: Gastropoda) as a bioindicator for endocrine disrupters in terms of steroid levels (testosterone (T) and 17b-estradiol (E)), alteration of microsomal CYP4501B1-like immunoreactivity, total protein (TP) level, and gonadal structure after exposure to sublethal concentrations of glyphosate for 3 weeks.
In order to study the ability of the snails' recuperation, the exposed snails were subjected to a recovery period for 2 weeks.

Results:

- level of T, E, and TP contents were significantly decreased ($p \leq 0.05$) in glyphosate-exposed groups compared with control (unexposed) group.
- level of microsomal CYP4501B1-like immunoreactivity increased significantly ($p \leq 0.05$) in glyphosate-exposed snails.
- Histological investigation of the ovotestis showed that glyphosate caused degenerative changes including azoospermia and oocytes deformation. All the recovered groups did not return back to their normal state.

It was concluded by the study authors that glyphosate was endocrine disrupter and causes cellular toxicity indicated by the decrease of protein content and the increase in CYP4501B1-like immunoreactivity. This toxicity is irreversible and the snail is not able to recover its normal state. The fluctuation of CYP4501B1 suggests that this vertebrate-like enzyme may be functional also in the snail and may be used as a biomarker for insecticide toxicity.

This study generated a 24 h LC50 value of 41.6 ppm in the snail *B. alexandrina* which is relevant for the aquatic risk assessment. In this study only the formulation "Herfosate" was used and no pure active substance glyphosate. ''Herfosate'' contains 48% w/v of glyphosate IPA, and inert ingredients equal 52% w/v. No additional information is provided on the nature of these co-formulants. No analytical confirmation of test concentrations was performed. Results on mortality are only graphically presented and raw data are not presented.
In fig 1, mortality is ranged between probit 4-6.5, corresponding to approximately 15-95% mortality. However given that mortality are only graphically presented and raw data are not presented given the LC50 value should be considered with caution together with other available values in a weight of evidence.

In addition, according to the study authors, observations on cellular and tissue-level endpoints are relevant for the ED assessment. RMS considers this study as not relevant in the sense of the EFSA guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 since results are not based on the active ingredient but to a formulation different than the representative formulation. The dose tested being equivalent to LC10, lower concentrations would have been necessary for investigation of endocrine disruption properties. No analytical confirmation of test concentrations was performed (LC10 was targeted).

Overall, for risk assessment the study and LC50 are considered as less relevant but supplementary (uncertainty on co-formulant in formulation) and reliable with restrictions.
Regarding the observations related to endocrine disruption issues, they are not relevant for the assessment of active substance glyphosate as only a formulation was tested and not the active substance itself..

B.9.3. EFFECTS ON ARTHROPODS

B.9.3.1. Effects on bees.

B.9.3.1.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.3.1, CP 10.3.1
Report author	Dai, P. *et al*.
Report year	2018
Report title	The Herbicide Glyphosate Negatively Affects Midgut Bacterial Communities and Survival of Honey Bee during Larvae Reared in Vitro.
Document Source	Journal of agricultural and food chemistry (2018), Vol. 66, No. 29, pp. 7786-7793
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Effects of glyphosate on survival, developmental rate, larval weight, and midgut bacterial diversity of *Apis mellifera* were tested in the laboratory. Larvae were reared *in vitro* and fed diet containing glyphosate at 0.8, 4, and 20 mg/L. The dependent variables were compared with negative control and positive control (dimethoate 45 mg/L). Brood survival decreased in 4 or 20 mg/L glyphosate treatments but not in 0.8 mg/L, and larval weight decreased in 0.8 or 4 mg/L glyphosate treatments. Exposure to three concentrations did not affect the developmental rate. Furthermore, the intestinal bacterial communities were determined using high-throughput sequencing targeting the V3−V4 regions of the 16S rDNA. All core honey bee intestinal bacterial phyla such as Proteobacteria (30.86%), Firmicutes (13.82%), and Actinobacteria (11.88%) were detected, and significant changes were found in the species diversity and richness in 20 mg/L glyphosate group.

Materials and methods

Glyphosate
Glyphosate (product number P109919-250 mg, purity 99.5%) was purchased from Aladdin, Inc. (Qigang Rd. Fengxian, Shanghai, China).

Honey Bee Rearing Conditions
All honey bees were obtained from the Institute of Apicultural Research apiaries (40°00′28″N, 116°12′18″E), Chinese Academy of Agricultural Sciences, in Beijing during June-August 2017. The colonies were of mixed race, *Apis mellifera*, housed in standard Langstroth-style equipment, and managed per common best management practices for the region. Honey bee larvae were reared *in vitro*. The authors' discussion of the *in vitro* timeline corresponds to Schmehl et al.'s Table 3, column 3, where all time points from grafting as day D = 0 or D0 are discussed. Honey bee queens were caged on a wax comb (D-4) for 24 h to lay eggs. At D0 (75 h after the queens were released), the larvae were transferred

from the comb to sterile, 48-well tissue culture plates (STCPs) with 20 µL of diet A (royal jelly 44.25%, glucose 5.3%, fructose 5.3%, yeast extract 0.9%, and water 44.25%) prepared in each well. On D2 (48 h after grafting), each larva was fed 20 µL of diet B (royal jelly 42.95%, glucose 6.4%, fructose 6.4%, yeast extract 1.3%, and water 42.95%). On D3, 4, and 5, each larva was fed 30 µL, 40 µL, and 50 µL, respectively, of diet C (royal jelly 50%, glucose 9%, fructose 9%, yeast extract 2%, and water 30%). The larval STCPs were placed horizontally in a larval growth chamber maintained at 94% R.H. and 35°C. Larvae were transferred from the larval STCP to the prepared pupal STCP when all available diet had been consumed (as early as D6). Pupal STCPs were maintained at 75% R.H. and 35°C. Adult worker bees began to eclose as soon 18 days after grafting. Emerging adults were collected at least twice daily and were maintained in hoarding cages with ad libitum access to pollen and 50% sugar water solution (w/v).

Experimental Design
Glyphosate was dissolved in water to prepare stock solution, and the solvent accounted for 0.5% of the volume of the final diets. The following treatments were conducted: glyphosate 0.8, 4, and 20 mg/L, negative control and positive control (45 mg/L dimethoate). Four replicates were conducted for each treatment. Larvae tested within each replicate were sourced from a single colony and each replicate was sourced from a different colony. A surplus of larvae was grafted for each replicate. On D2, a minimum of 16 robust larvae per replicate were randomly selected for each treatment group and fed 20 µL of diet B containing the test solution appropriate to the group's assigned treatment. On D3, 4, and 5, the larvae were fed 30, 40, and 50 µL, respectively, of diet C containing the appropriate test solution.

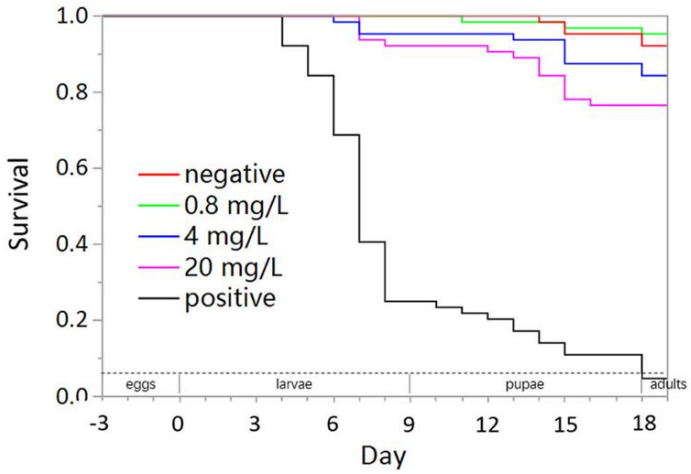
End-Points
Larval survival was noted by viewing larvae under a dissecting microscope at which time spiracular movement (opening / closing) was noted. The individual was considered dead if no spiracular movement was detected. Pupal survival was monitored daily by visual inspection of the pupae. Dead prepupae and pupae were recognized by occasional black or white subdermal necrotic stains or visible wilting. Any larvae or pupae determined to be dead were removed from the plates. Survival rates was assessed each day for each treatment group. Additionally, the developmental rate was calculated for each treatment. Furthermore, larval fresh body weight at D6 was calculated for each larva immediately prior to transfer to the pupal plate.

Statistical Analysis
Statistical analyses were performed using the SAS 9.2 software program (USA). The survival rate data were tested with a Kaplan-Meier analysis. Furthermore, ANOVAs and Tukey's HSD tests were used to compare developmental rate or larval weight at D6 among the experimental groups.

Results

Survival
Negative control average total survival was 92.2%, and positive control (45 mg/L dimethoate) average total survival was 5.0%. There were no statistical differences between survival of larvae fed 0.8 mg/L glyphosate and that of larvae fed the negative control diet (Figure 1). However, survival of larvae fed 4 mg/L or 20 mg/L of glyphosate was a statistically significant decrease compared to that of larvae fed the negative control diet. Survival of larvae fed 0.8 mg/L, 4 mg/L or 20 mg/L of glyphosate was significantly higher than those fed positive control diet.

Figure 1. Total survival of honey bees exposed to glyphosate during larval development on D2 thru D5 after grafting (*N* = 4 replicates of 16 larvae/replicate, or 64 larvae, per test substance). Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control and no contaminated diet as a negative control. D18 on the figures corresponds to D21 from egg laying to adult emergence for the honey bee.



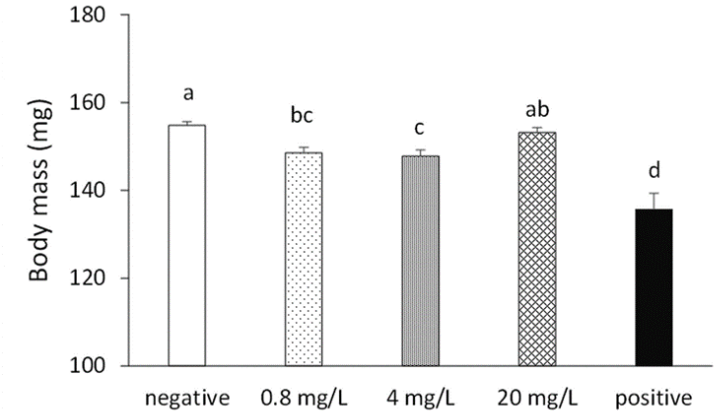
Developmental Rate

A one-way ANOVA was conducted to compare the effect of glyphosate on the developmental rate of larvae, pupae, and both combined (total). Exposure to glyphosate did not affect the larval, pupal, and total developmental rate.

Larval Weight

A one-way ANOVA was conducted to compare the effect of glyphosate on larval weight on D6. Exposure to 0.08 or 4 mg/L glyphosate significantly decreased the larval weight (Figure 2).

Figure 2. Body weight on D6 of honey bee larvae reared in vitro and exposed to glyphosate in the diet on D2 thru D5 after grafting. Larvae were fed a dimethoate-contaminated diet (45 mg/L) as a positive control or no contaminated diet as a negative control. Bars with the same letter are not different at $\alpha \leq 0.05$.



Conclusion

Honey bee larvae may be exposed to glyphosate orally given their diets contain pollen and honey. On the basis of the concentrations recommended for spraying and on those measured in natural environments, the effects of glyphosate to honey bee larvae reared in vitro were investigated. The results showed that brood survival was significantly lower for individuals fed diet with 4 mg/L and 20 mg/L glyphosate than for ones fed the negative control diet, and exposure to 0.08 or 4 mg/L glyphosate significantly decreased the larval weight. No adverse effects on brood developmental rate were observed

in any of the glyphosate treated larvae. Pollen and honey/nectar represent only a small part of larval diet. It seems unlikely that the glyphosate levels found in brood food will approach the maximum residues found in pollen or nectar/ honey under normal environmental conditions.

Glyphosate 4 mg/L and 20 mg/L chronic exposure decreased brood survival and glyphosate 20 mg/L affected gut bacterial communities of newly emerged bees.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The bacterial communities in the mid-gut of bees were characterised. No gut bacterial analysis was conducted on the positive control bees. Overall an increase in abundance and richness of bacterial taxa was observed at the highest exposure concentration. The implications of this was not discussed in the paper. Bacterial assemblages in the gut of honey bees is not relatable to an EU level ecotoxicologal regulatory risk assessment. The study is adequately described including specifications of the test item and test design. However, no regulatory endpoints were derived and there is no analytical verification of dose solutions.

Assessment and conclusion by RMS:

Effects of glyphosate on survival, developmental rate, larval weight, and midgut bacterial diversity of *Apis mellifera* were tested in the laboratory. Larvae were reared in vitro and fed diet containing glyphosate 0.8, 4, and 20 mg/L. Brood survival decreased in 4 or 20 mg/L glyphosate treatments but not in 0.8 mg/L, and larval weight decreased in 0.8 or 4 mg/L glyphosate treatments. Exposure to three concentrations did not affect the developmental rate. The intestinal bacterial communities showed significant changes in the species diversity and richness in 20 mg/L glyphosate group.

RMS considers this study partly relevant (for brood survival and development).
RMS considers that the study was well conducted (study design is deemed correct).
Validity criteria (OECD 239) are fulfilled.

RMS notes that the concentrations inducing adverse effects on brood survival (4 or 20 mg/L glyphosate) were below those issued from regulatory studies. In an other study ([redacted], 2020, CA 8.3.1.3/001) NOEC was 505 mg a.s./kg diet (NOED was 80 µg a.s./larva). The reason of such difference is not clear but RMS cannot discard a difference of toxicity between the test items that were tested. Indeed, in this study glyphosate was tested, although the other study ([redacted] 2020, CA 8.3.1.3/001) used an IPA salt of glyphosate.

On an other hand, the statistics should be considered with caution as the magnitude of effects appears low. By day 18, significant effects were observed on survival in 4 or 20 mg/L treatments. Although the values were not reported (only the graphical representation was given), survival of ~85 and ~75% can be inferred from the figure for 4 or 20 mg/L groups, respectively. Although the effects were statistically significant, they may be caused by natural variation – one of the validity criteria for the test (OECD Series on Testing and Assessment No. 239) is a minimum 70% emergence (i.e. survival) by day 22. Since the survival in all treatments was above 70%, the significancy of the effects should be considered with caution.
The results of this study (brood survival) are considered relevant and reliable with restrictions by RMS. They may entail a need to consider the glyphosate forms in the products undergoing a marketing procedure but overall, no evidence of adverse effects on survival is found in this study.

The authors stated that the effects on larval fresh weight were observed on day 6 in 0.8 or 4 mg/L glyphosate treatments, but not in 20 mg/L glyphosate treatment. RMS considers the differences minor and the effect was not dose related. Therefore, the slight reduction of larval weight is not considered treatment related.

In this study bacterial communities in the mid-gut of honeybees were affected. The results on this parameter are not relevant for regulatory risk assessment. Indeed, there is no clear conceptual link between bacterial communities in the mid-gut of honeybees and the specific protection goals for bees (SPG). RMS agreed that it may play a role in the colony/population health, but such link is not immediate in conceptual terms and not quantifiable. Moreover, the results were obtained only from five individuals per treatment which is considered insufficient to account for natural variation. Therefore, the results are considered unreliable. The corresponding part of the summary was deleted by RMS as considered not directly relevant and not reliable.

The study is considered relevant and reliable with restrictions for brood survival and development part.

B.9.3.1.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

No study belong to this category. However, datagap has been identified.

B.9.3.2. Effects on non-target arthropods other than bees

B.9.3.2.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

No study belong to this category. However, datagap has been identified.

B.9.3.2.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Mirande L. *et al*.
Report year	2010
Report title	Side-effects of glyphosate on the life parameters of Eriopis connexa (Coleoptera: Coccinelidae) in Argentina
Document No	Communications in Agricultural and Applied Biological Sciences, (2010) Vol. 75, No. 3, pp. 367-72
Guidelines followed in study	None
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by title/abstract

Full summary of the study according to OECD format

In Argentina, transgenic soybean crop (Roundup Ready, RR) has undergone a major expansion over the last 15 years, with the consequent increase of glyphosate applications, a broadspectrum and post emergence herbicide.
Soybean crops are inhabited by several arthropods. *Eriopis connexa* Germar (Coleoptera: Coccinelidae) is a predator associated to soybean soft-bodies pest and have a Neotropical distribution. Nowadays, it is being considered a potentially biological control agent in South America.
The objectives of this work were to evaluate the side-effects of glyphosate on larvae (third instar) and adults of this predator. Commercial compound and the maximum registered concentrations for field use were employed: GlifoGlex 48® (48% glyphosate, 192 mg a.i./litre, Gleba Argentina S.A.). The exposure was by ingestion through the treated prey (*Rophalosiphum padi*) or by drinking treated water during 48h for treatment of the adult. The herbicide solutions were prepared using distilled water as solvent. The bioassays were carried out in the laboratory under controlled conditions: 23±0.5°C, 75±5% RH and 16:8 (L:D) of photoperiod. Development time, weight of pupae, adult emergence, pre-oviposition period, fecundity and fertility were evaluated as endpoints.
Larvae from glyphosate treatment molted earlier than controls. In addition, the weight of pupae, longevity, fecundity and fertility were drastically reduced in treated organisms. The reductions were more drastic when the treatments were performed at the third larval stage than as adult. The reproduction

capacity of the predator was the most affected parameter and could be related to a hormonal disruption by glyphosate in the treated organisms.
This work can confirm the deleterious effects of this herbicide on beneficial organisms. Also, it agrees with prior studies carried out on other predators associated to soybean pest, such as *Chrysoperla externa* (Neuroptera: Chrysopidae) and *Alpaida veniliae* (Araneae: Araneidae).

Materials and methods

Insects
Adults and larvae of *E. connexa* were collected in the field (La Plata, Argentina) in crops or wild areas with no history of pesticide use. The collected organisms were maintained under quarantine for discarding parasitism. The colony was initiated with the progeny from field-collected females and maintained in the laboratory during two generations prior to bioassays. The rearing and bioassays conditions were: 23±0.5°C, 75±5% RH and a 16:8 (L:D) photoperiod. *E. connexa* were fed with nymph and adults of *Rophalosiphum padi* L. ad libitum as prey.

Bioassays
Commercial compound and the maximum registered concentration for their field use were employed: GlifoGlex 48® (48% glyphosate, 192 mg a.i./litre, Gleba Argentina S.A.). The glyphosate solution was applied by the drinking of treated water for the treatment of adults or by ingestion through the treated prey (*R. padi*) to larvae (at third larval instar, ≤24 h-old) during 48 h, respectively. The prey was dipped in the herbicide dilution during 5 s, dried at room conditions and offered to larvae. Distilled water was used as dissolvent and a tensioactive (0.02% Tween 80®, Merck) was added to stick the herbicide to prey. Preys for control treatments were dipped in distilled water + tensioactive alone. Twenty replicates of one larva or adult were used per treatment, respectively.
The endpoints were: the development time of immature stages, weight of pupae, adult longevity, fecundity and fertility of females. The fecundity and fertility of 5-days-old mated females were evaluated during the first five ovipositions.
Eggs of 24 and 48 h old from both treatments were prepared for observation under the light stereomicroscope (Leica MZ6, Leica, Wetzlar, Germany) with a cold light. These specimens were fixed in Bouin's® solution, dehydrated in alcohol series (from 20-100 % v/v) and mounted on a glass microscope slide in Hoyer®. Abnormalities and development of embryos were evaluated.

Statistical analysis
The data are presented as mean ±SE and analyzed by ANOVA or Kruskal-Wallis test. Accordingly, means or medians were compared by LSD or Box and Whisker plot, respectively. The Statgraphics Program version 5.0 was used.

Results

No mortality was observed in the organisms exposed in the short-effect bioassay to glyphosate. However several long-term effects were recorded in survivors. As depicted in Figure 1A, larvae from the glyphosate treatment molted earlier than the controls. Moreover the herbicide significantly reduced the weight gained from L3 to L4 instars (F= 73.33; df=1,38; P<0.01).
In addition, treatment with the herbicide reduced significantly the longevity of adults emerged from treated immature stage (as L3 instar), and had more deleterious effects when the exposition was done at the third larval instar than at the adult stage (F= 116.8; df 1,78; P ≤0.001; K=11.72; P=0.0006) (Figure 2). The adults lived 20-40 days less long in the glyphosate treatment than in the controls.
As shown in Figure 3, the fecundity and fertility were negatively affected by the herbicide. The fertility was also more drastically reduced when the treatment was done during the larval stage (only 10.53% of the eggs hatched successfully). The reduction of this parameter was higher than 80% with respect to control. The eggs laid by females from the glyphosate treatment turned black and dehydrated two days after being laid. In the glyphosate treatments, typically there was no embryo detected inside of the chorion of 24 h-old eggs (Figure 4).

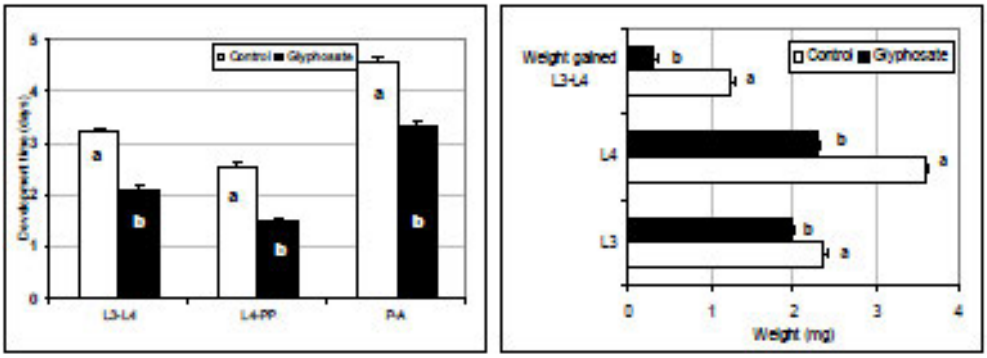


Figure 1. Effects of glyphosate on the intermolt development time (A) and weight (B) of *Eriopis connexa* exposed to the herbicide by ingestion (treated prey) at third larval instar.

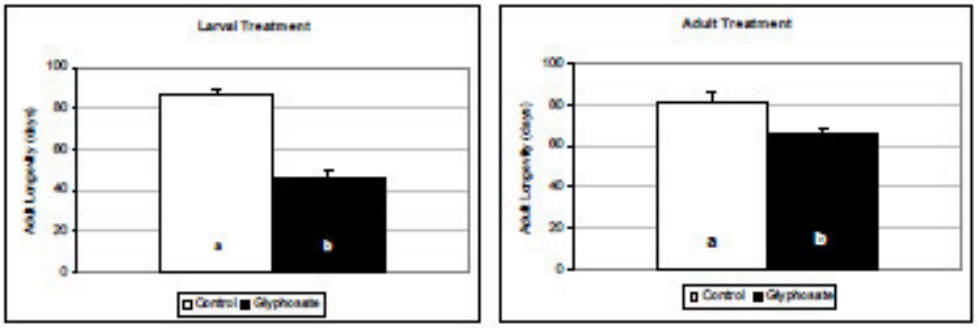


Figure 2. Sublethal effects of glyphosate on the adult longevity of *E. connexa*. Larval treatment: (Fecundity: F=458.62; df 1,28; P≤0.001; Fertility: F=386.87; df 1,28; P≤0.001). Adult treatment: (Fecundity: K=14.21; P=0.0001; Fertility: F=441.96; df 1,18; P≤0.001).

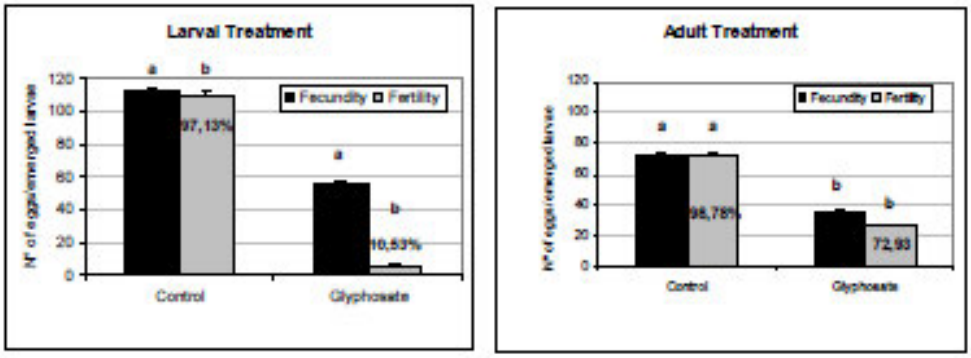


Figure 3. Sublethal effects of glyphosate on the fecundity and fertility of *E. connexa* females exposed to the herbicide by ingestion (treated prey) at third larval instar.

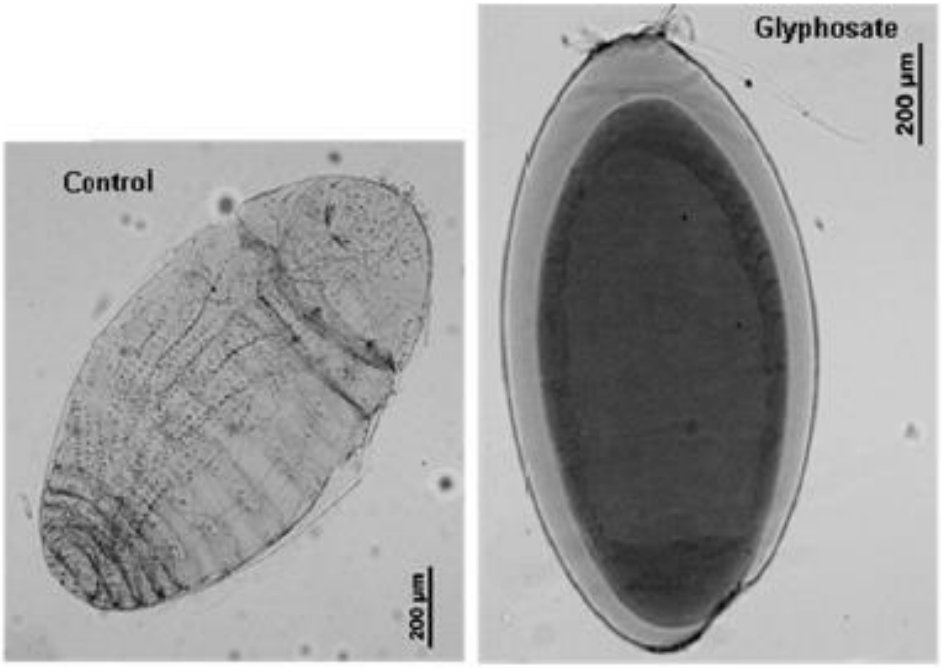


Figure 4. Eggs of *E. connexa* (48 h-old) from control females and exposed to the herbicide by ingestion (treated prey) at third larval instar. Embryo is visible in the control, whereas in the egg from glyphosate no embryo was observed and darkness could be related with the death of it.

Conclusion

The present study demonstrated that the maximum field recommended concentration of glyphosate (192 mg a.i./liter) caused several long-term effects in the organisms treated. The reproduction capacity of the predator was the most affected parameter and this can be related to a hormonal disruption by glyphosate in the treated organisms. Likewise, several negative disruptions were detected in the development time, immature stage weight and adult longevity. These side-effects can negatively affect the fitness of *E. connexa*, reducing drastically its role as a natural mortality factor of soybean pests. It agrees with prior studies carried out on other predators associated to soybean pest, such as *Chrysoperla externa* (Neuroptera: Chrysopidae) (Schneider et al., 2009) and *Alpaida veniliae* (Aranea: Araneidae) (Benamú et al., 2010).

Based on these studies glyphosate resulted to be harmful toward *E. connexa*, causing development, morphological, physiological, biochemical and immunological alterations.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by title/abstract: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). In addition, the tested formulation GlifoGlex 48 is not the representative formulation for the glyphosate EU renewal, thus the article is not relevant.

Further points of clarification:

The representative formulation is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.

Assessment and conclusion by RMS:

The objectives of this work were to evaluate the side-effects of glyphosate on larvae (third instar) and adults of *Eriopis connexa* Germar (Coleoptera: Coccinelidae).
GlifoGlex 48® (48% glyphosate) was used. This study is therefore considered of limited relevance for the assessment of glyphosate itself as no information was provided on the surfactants present in the formulation.
The concentration of 192 mg a.i./litre is considered realistic and even below the concentration that may be sprayed under realistic conditions of use.
The exposure was by ingestion through the treated prey (*Rophalosiphum padi*) or by drinking treated water during 48 h for treatment of the adult, both ways are considered relevant by RMS.
Development time, weight of pupae, adult emergence, pre-oviposition period fecundity and fertility are all regarded as relevant parameters by RMS.
Overall, the relevance of this study is limited due to the uncertainty around the co-formulants present in the formulation.

Larvae from glyphosate treatment molted earlier than controls. In addition, the weight of pupae, longevity, fecundity and fertility were drastically reduced in treated organisms. The reductions were more drastic when the treatments were performed at the third larval stage than as adult. The reproduction capacity of the predator was the most affected parameter and it was deemed to be related to a hormonal disruption by glyphosate in the treated organisms.

No raw data is available, only graphics are presented. The study seems nevertheless to have performed well and adverse effects are obvious.
The study is reliable with restrictions.

B.9.4. EFFECTS ON NON-TARGET SOIL MESO- AND MACROFAUNA

B.9.4.1. Earthworm – sub-lethal effects

B.9.4.1.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.4.1/005 also referenced under CA 8.4.2.1/005 and CA 8.5/005
Report author	von Mérey, G. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil biota
Document No	DOI: 10.1002/etc.3438 E-ISSN: 1552-8618
Guidelines followed in study	OECD 222; OECD 226; OECD 232; OECD 216
Deviations from current test guideline	Earthworm cocoons were not counted, in accordance with OECD 222.
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability (RMS):	Yes/Reliable This publication actually corresponds to the regulatory studies summarized and already assessed by RMS

	CA 8.4.1/001 [redacted], 2009 Glyphosate IPA salt Eisenia andrei CA 8.4.1/003 [redacted], 2003 AMPA Eisenia fetida CA 8.4.1/001 [redacted], 2010 Glyphosate IPA salt Folsomia candida CA 8.4.1/002 [redacted], 2009 Glyphosate IPA salt Hypoaspis aculeifer CA 8.4.2.1/003 [redacted] 2010 AMPA Folsomia candida CA 8.4.2.1/004 [redacted] 2010 AMPA Hypoaspis aculeifer CA 8.5/001 [redacted] 2014 Glyphosate Nitrogen transformation CA 8.5/004 [redacted],, 2010 AMPA Nitrogen transformation Therefore, this publication was not assessed by RMS.

Assessment and conclusion

Assessment and conclusion by applicant:

The aim of the paper was to evaluate potential effects of Glyphosate, Glyphosate salt and AMPA on earthworm, soil mites, springtails and soil micro-organisms.

The studies have been conducted according to recognised guidelines and validity criteria were presented. Test substance information, test organism origin, study designs and toxicity effects were adequately described. The study is considered reliable.

Assessment and conclusion by RMS:

This publication actually corresponds to the regulatory studies summarized and already assessed by RMS
CA 8.4.1/001 [redacted], 2009 Glyphosate IPA salt Eisenia andrei
CA 8.4.1/003 [redacted], 2003 AMPA Eisenia fetida
CA 8.4.1/001 [redacted], 2010 Glyphosate IPA salt Folsomia candida
CA 8.4.1/002 [redacted] 2009 Glyphosate IPA salt Hypoaspis aculeifer
CA 8.4.2.1/003 [redacted] 2010 AMPA Folsomia candida
CA 8.4.2.1/004 [redacted] 2010 AMPA Hypoaspis aculeifer
CA 8.5/001 [redacted] 2014 Glyphosate Nitrogen transformation
CA 8.5/004 [redacted],, 2010 AMPA Nitrogen transformation

Therefore, this publication was not assessed by RMS.

Data point:	CA 8.4.2
Report author	Correia F. V. *et al*.
Report year	2010
Report title	Effects of glyphosate and 2,4-D on earthworms (*Eisenia foetida*) in laboratory tests.
Document Source	Bulletin of environmental contamination and toxicology (2010), Vol. 85, No. 3, pp. 264-268
Guidelines followed in study	ISO 11268-1, 1993 ISO 11268-2, 1998

Deviations from current test guideline	Validity criteria cannot be checked
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

Laboratory tests were conducted to compare the effects of various concentrations of glyphosate on earthworms (*Eisenia foetida*) cultured in Argissol during 56 days of incubation. The effects on earthworm growth, survival, and reproduction rates were verified for different exposure times. Earthworms kept in glyphosate treated soil were classified as alive in all evaluations, but showed gradual and significant reduction in mean weight (50%) at all test concentrations. No cocoons or juveniles were found in soil treated. Glyphosate demonstrated severe effects on the development and reproduction of *Eisenia foetida* in laboratory tests in the range of test concentrations.

Materials and methods

Test item: Glyphosate, purity 99.7%, was obtained from Sigma Chemical.

Soil samples used in the experiments were collected in the experimental area of Embrapa Agrobiologia, located in the municipal district of Seropedica (Rio de Janeiro) and classified as Argissol. The source was selected as one of the most representative soils in Brazilian territory. The soil is used extensively in corn, soy, sugarcane, and other crops in tillage and non-tillage systems. The soil samples showed the following characteristics: 608 g/kg sand; 112 g/kg silt; 280 g/kg clay; 10.8 g/kg organic carbon; and pH 5.5.

The *Eisenia foetida* earthworms were obtained from the Minhocario Arborium (RJ). They were carefully transported to the laboratory and acclimatized for 4 weeks before any experiment, at 20°C ± 2, in bovine manure. All tests were performed using adult earthworms (age less than 2 months and clitella well developed) and with individual weight from 300 to 600 mg (ISO 11268-1, 1993).

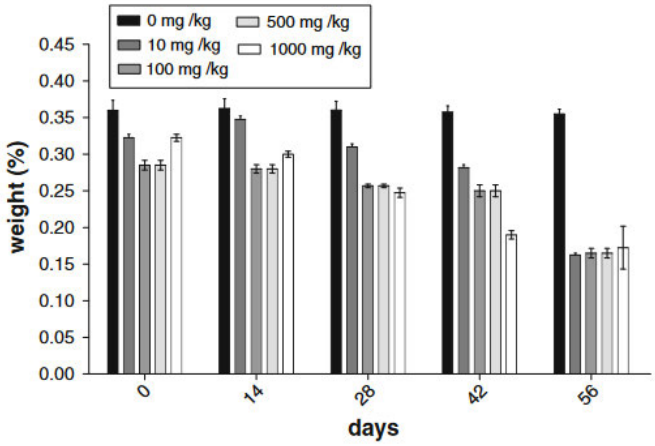
For the experiments, 10 earthworms were transferred to each container with 400 g of soil prepared by adding different concentrations of glyphosate (dry weight basis). Four replicates were analyzed for each concentration and 4 control containers, prepared under identical conditions without the addition of the target pesticide. The concentrations used were 1; 10; 100; 500; 1000 mg/kg. The containers were covered with paper-filter with holes to maintain aeration conditions during the 56 days of the test. Soil moisture was standardized at 60% of maximum water-holding capacity, and the samples were maintained at room temperature (20 ± 2°C) in the presence of light. During all the experiments, moisture content was checked and maintained at 60% by adjusting the weight of the container against the weight known from the previous week prior to sampling. After 14, 21, 28, 42, and 56 days of incubation, the containers were opened, surviving and dead earthworms were counted, and the survivors' average weight was verified. Earthworms were classified as dead when they did not respond to a gentile mechanical stimulus and morphological abnormalities were recorded. For the reproduction test, cocoon production and numbers of hatched juveniles were hand-sorted, returned, and further incubated during the experiment (ISO 11268-2, 1998).

All the data were analyzed by ANOVA using Tukey's test for comparing the treatments and duration of exposure for each herbicide and control groups. The results were expressed as means ± SD (standard deviation). Statistical significance was set at $p < 0.05$.

Results

In the growth test, earthworms from the soil treated with glyphosate showed a gradual reduction in mean weight during the experiment. The effect was observed at all test concentrations when compared to untreated soil (Fig. 1). The percentage of weight loss at the end of the experiment was approximately 50% of baseline weight. All earthworms were classified as alive at all moments of sampling. The growth rates of untreated worms were considered non-significant during the 56 days of the experiments. The average biomass of untreated worms was the same. In contrast, worms from glyphosate-treated soil samples, in terms of time dependency, were significantly different ($p < 0.05$) from the controls, whereas no concentration dependency was observed with this chemical.

Figure 1. Effect of glyphosate soil concentration and exposure time on *Eisenia foetida* biomass



Although glyphosate did not kill the test organisms in the range of test concentrations, the decrease in mean weight may indicate a chronic effect of this herbicide. The reduction in mean weight of worms exposed to soils treated with glyphosate (50%) can be explained by the morphological changes caused by the herbicides.

Significant anatomical changes were observed at 30 days. Morphological abnormalities like elevating the body, coiling, and curling were observed in all specimens exposed to the highest concentrations of glyphosate after 30 days of exposure. Similar abnormalities were also observed in the organisms exposed for 50 days at lower concentrations. Excessive mucous secretion was noticed in all exposed worms. The exposed worms were also much less active than the controls. In soil treated with glyphosate, morphological changes (ruptures in the body wall and bloody lesions) were also observed in the midsection of the earthworm bodies. Fragmentation of the body was also observed as a result of these changes. Fifty percent of all treated worms developed breakages and shedding of cuticle (dried), which resembles ecdysis (shedding of skin) in insects and snakes.

Table 1 shows the results of the reproduction tests in the soils treated with glyphosate. No cocoons or juveniles were found in any experiment using soil containing the target herbicide. Reproduction was always lower than 20% when compared to the control, so that according to Dechema (1995), it can be classified as toxic. Despite the low reproduction observed in the controls, the absence of cocoons or juveniles in soils treated with glyphosate may result from interference by these substances in the earthworms' reproductive mechanism. The significant reduction ($p < 0.05$) in cocoons and juveniles was observed in all treated worms, compared to untreated worms. Additional studies are required to fully understand this effect. In addition, loss of weight was observed in the earthworms at the end of the experiment as compared to the controls, suggesting a significant toxic effect.

Table 1. Mortality (% of dead animals related to numbers of tested specimens) and reproduction test (total numbers of cocoons (28d) and hatched juveniles (56d)). These results were obtained from 4 replicates (n=40worms).

Conc. mg/kg	Mortality (%)	Reproduction test	
		Cocoons	Juveniles
Glyphosate			
0	0	7	4
10	0	0	0
100	0	0	0
500	0	0	0
1,000	0	0	0

Soil ingestion and dermal absorption are the most important intake routes of soil pollutants by earthworms. In the present study, no avoidance behavior was observed in soils treated with glyphosate.

Conclusion

Glyphosate demonstrated severe effects on the development and reproduction of *Eisenia foetida* in laboratory tests. Long-term exposure (56 days) to soil contaminated with glyphosate demonstrated a toxic effect on normal development and reproduction of *Eisenia foetida*, indicating that this substance may have significant toxic effects on soil biota.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study looks at the impact of the active substance glyphosate on earthworm reproduction and was conducted to relevant guidelines. Test concentrations were from 1 to 1000 mg/kg in a 56 day study. Data is useful but there is no reliable endpoint to be used in the EU ecotoxicological regulatory risk assessment / glyphosate EU renewal. In addition, Brazilian soils were used in the study. Such conditions can be difficult to extrapolate to EU (e.g. different geo-climatic properties, land-uses and agricultural practices etc.).

Further points of clarification:
Validity criteria for reproduction in the control group was not satisfied (to achieve ≥ 30 juveniles by the end of the test).

Assessment and conclusion by RMS:
The study assess the effects of the active substance glyphosate on earthworm reproduction. Thus the study is relevant for risk assessment purpose.

This study did not follow OECD 222 recommendations. Mortality and growth effects on the adult worms were determined up to 56 days instead of 28 d (OECD 222). The authors indicated to have followed ISO 11268-2. The method is therefore suitable for assessing effects on eartworms reproduction.
Earthworms kept in glyphosate treated soil were all alive at all concentrations (up to 1000 mg/kg), but showed gradual and significant reduction in mean weight (50%) at all test concentrations. Morphological abnormalities like elevating the body, coiling, and curling were observed in all specimens.
No cocoons or juveniles were found in treated soils (very few in control, less than ≥ 30 juveniles by the end of the test as required by OECD 222).
Brazilian soils were used in the study (this is not considered to affect the relevance of the study for the risk assessment).
Very low number of coccons/juveniles were produced in control. This questions the condition of the worms in the study.
The results on reproduction are not reliable.
A NOEC for mortality of 1000 mg/kg soil could be derived.
No NOEC can be established as effects on bodyweight were noted at all concentrations (from 10 mg/kg to 1000 mg/kg). LOEC = 10 mg/kg
Overall, this study is relevant and reliable with restrictions.

B.9.4.1.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 8.4, CP 10.4.2.2
Report author	Santos M. J. G. et al.
Report year	2012
Report title	Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei.
Document Source	Ecotoxicology (2012), Vol. 21, No. 8, pp. 2113-2122
Guidelines followed in study	ISO 17512-2, ISO 11268-2 and ISO 11267 guidelines for laboratory phase
Deviations from current test guideline	No major deviations
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

The objective of this work was to assess the impact of pesticide application to non-target soil organisms simulating what happens following pesticide application in agricultural fields and thus obtaining higher realism on results obtained. For that purpose, a glyphosate-based commercial formulation Montana® (30.8 % a.i. glyphosate) was applied to a Mediterranean agricultural field. The soil was collected after

spraying and dilution series were prepared with untreated soil to determine the impact of the pesticide on the avoidance behaviour and reproduction of the earthworm *Eisenia andrei* and the collembolan *Folsomia candida*. Glyphosate based formulation Montana® did not seem to affect either earthworms or collembolans in the recommended field dose.

Materials and Methods

Pesticide field application

The field trial was performed in the Low Mondego Region. The field had been left fallow for more than 6 years, thus the site soil was assumed to be free of pesticide residues. Prior to pesticide application a composite soil sample of the study site was collected to perform a brief soil characterization: grain size distribution - 62.8% coarse sand, 25.9% fine sand, 7% silt, 4.2% clay; pH (H_2O) = 7.48; pH (KCl) = 7.31; bulk density = 2.4 g/cm^3; water holding capacity = 70%; cation exchange capacity = 8.86 meq/100 g; organic matter content = 2.4%. A commercial formulation of glyphosate Montana® (30.8% a.i. glyphosate) was used in this study. The FOCUS guidance documents (FOCUS 2000) were used for calculation of the plateau concentration and annual cumulative application dose, considering crop interception levels, soil density and pesticide degradation rates. Two weeks prior to pesticide application, plant cover was cut, and the soil was harrowed. A replicated plot design (5 replicates per treatment) was used, pesticides were sprayed onto 20 plots (control, where water was applied instead of pesticide, plus four pesticide treatments) of 25 m^2 (5 x 5 m) randomly distributed within the testing area and separated by 2 m-wide corridors in order to avoid cross-contamination. The plateau concentration was applied using a PL1 sprayer equipped with ten flat ray spray nozzles and the chemicals were incorporated into the soil (10 cm) by surface harrowing. Two days after the application of the plateau concentration, and using the same technique, the recommended annual cumulative dose, i.e. the sum of all applications of the plant protection product within a year of each pesticide, was sprayed onto the corresponding replicated plots, simulating a 'worst case scenario'. Pesticide incorporation was assured by simulating a rain event. Two days after the pesticide application, soil samples were collected from each replicated plot (first 10 cm of soil). Samples were transported to the laboratory and stored at -20 °C until further analysis.

Pesticide extraction and analysis

The glyphosate concentration was determined in the corresponding soil samples and in control samples according to the method of Aubin and Smith (1992). Five grams of soil were extracted 2 times with 20 mL of 0.1 M NaOH in a centrifuge tube. The mixture was shaken, then centrifuged and decanted. The collected extracts were filtered, neutralised with HCl and the extract volume was reduced in a rotavapor and later cleaned up using a 500 mg C-18 cartridge. Extracts were then derivatized and analyzed by HPLC–UV. The injection volume was 20 µL and acquisition was performed at 240 nm. Only LC grade solvents from Labscan were used in all analytical steps. All pesticides stock standards were purchased commercially. Glyphosate calibration standards were prepared in ultra-pure water.

Test organisms

The collembola *Folsomia candida* and earthworm *Eisenia andrei* were obtained from laboratory cultures, maintained at a constant light regime of 16 h light : 8 h dark and a constant temperature of 20 ± 2 °C. The earthworms were maintained in plastic boxes with a mixture of *Sphagnum* peat and horse dung (1:1, v:v) as substrate, adjusted to pH 6-7 with $CaCO_3$. The individuals used in the tests were more than one month old, clitellated, and with an individual fresh weight between 200 and 600 mg. The springtails were cultured in plastic boxes lined with a mixture of plaster of Paris and activated charcoal in a ratio of 9:1. On a weekly basis granulated dry yeast was added as food in small amounts to avoid spoilage by fungi. The experiments for the reproduction tests were done with juveniles 10–12 days old.

Ecotoxicological studies

Soil preparation

Following pesticide application, the 5 samples of pesticide treated plots were mixed and sieved. The soil was moistened to 60% of the water holding capacity for all the tests, and the pH was measured at the beginning and end of each test. Pesticide was tested in four dilutions (plus control): 25, 50, 75 and 100%

of the recommended application dose were prepared through mixing of soil from treated plots with soil from control plots to obtain the different dilutions.

Avoidance tests with earthworms
The avoidance assays with earthworms were made based on the ISO guideline 17512-2 (ISO 2007a). Each plastic container was divided into two equal sections with a plastic card, one-half of the container received 250 g (dw) of control soil (section A) and the other half 250 g (dw) of agricultural soil contaminated with the pesticide (section B). All combinations of contaminated/uncontaminated soil were tested, each one with five replicates. After placing the soils into each container, the card divider was removed and 10 worms were placed on the middle line. Afterwards, each container was covered with a transparent lid perforated for aeration. The organisms were incubated at 20 ± 2 °C with a photoperiod of 16 h : 8 h (light:dark) for 2 days. After the test period, the divider was put back to separate the control and test soils, and the number of worms in both sections were counted. To evaluate mortality rate, a spatial bias test (dual control avoidance test) was performed, in which both sections of the test containers were filled with the same soil dilution.

Avoidance tests with collembolans
The avoidance assays were made based on the guideline ISO 17512-2 (ISO 2007b). Therefore, 30 g (dw) of soil were placed in each half of cylindrical plastic container with a plastic divider. After removing the plastic divider, 20 individuals of *F. candida* were placed in the middle line of each test container. Five replicates were used for each combination tested. Test containers were incubated at 20 ± 2 °C with a photoperiod of 16 h:8 h (light:dark) for 2 days. At the end of the testing period, the control and test soils were carefully separated along the midline and each soil was emptied into small vessels. These were filled with water and with a few drops of blue ink. After gently stirring the vessel, the animals floating on the water surface were counted. In this avoidance procedure, also a dual control avoidance test was performed.

Reproduction tests with earthworms
The experimental setup was according to ISO guideline 11268-2 (ISO 1998). Five replicates per test concentration were prepared, each consisting of a cylindrical plastic container with approximately 500 g (dw) of soil. Ten clitellate earthworms were placed in each replicate. To prevent worms from escaping, the test vessels were covered with transparent lids with some holes for aeration. The test vessels were incubated in a controlled chamber at 20 ± 2 °C and a light:dark regime of 16 h : 8 h. Food (oat meal) was added to the surface of the test matrix at the beginning of the test and then once every 14 days during the experiment. The surviving earthworms were recorded after 28 days. The soil was returned to the vessels, incubated for another 28 days to continue the exposure of cocoons and juveniles to the test soil. At the end of the test, the number of hatched and non-hatched cocoons, the number of juveniles was recorded for each of the five replicates.

Reproduction tests with collembolan
The test was performed according to ISO guideline 11267 (ISO 1999). At the beginning of each experiment, 10 springtails were introduced in glass flasks containing 30 g of soil (dw). At the beginning of the test and after 14 days, approximately 2 mg of dry yeast was added to all test containers. Twice a week the test containers were opened to allow aeration, and water was added when necessary. After 28 days, the content of each test vessel was carefully transferred into larger vessels and filled up with water and some drops of blue ink. After moderate stirring, adults and juveniles floating on the water surface were photographed and counted using image analysis software provided by Leica.

Statistical analysis
Differences between the number of juveniles produced by earthworms or collembolans in the controls and the soil treatments were analysed using a one-way ANOVA, followed by post hoc Dunnett's test ($p < 0.05$). Prior to analyses data was checked for normality (Kolmogorov-Smironov test) and homogeneity of variances (Bartlett test). EC_{50} values were determined using appropriate nonlinear models. For all the analysis mentioned above, STATISTICA 7.0 software was used. The percentage of avoidance was calculated using the formula A = [(C-T)/N] x 100 (ISO 2007a), where A is the percentage of avoidance, C is the number of organisms in control soil, T is the number of organisms in the test soil (contaminated)

and N is the total number of organisms. The significance of the avoidance behaviour was tested using Fisher's exact test (Natal-da-Luz et al. 2009). EC_{50} values for avoidance behaviour (AC_{50}) were calculated with the probit method using the statistical software MINITAB 13 assuming that individuals were equally distributed over both sides of the test vessels (Loureiro et al. 2005). The concentration causing 50% of mortality (LC_{50}) was calculated with a logistic equation using the statistical software STATISTICA 7.

Results

Pesticide analysis

As expected, soil samples from control plots showed undetectable pesticide concentrations: glyphosate residues were below the detection level. For the calculation of the AC_{50}, EC_{50} and LC_{50} values the geometric mean of the concentrations measured in 5 replicates was used. Soil pH at the beginning and end of the tests varied between 7.01 and 7.87 in the soil contaminated with glyphosate.

Table 2: AC_{50}, EC_{50} and LC_{50} values values and 95 % confidence intervals calculated for the effect of MONTANA® on the avoidance behaviour and reproduction success of the earthworm *Eisenia andrei* and collembolan *Folsomia candida.*

Test organism	Commercial formulation	Active ingredient	AC_{50}	EC_{50}	LC_{50}
Eisenia andrei	Montana	Glyphosate	ND	ND	ND
Folsomia candida	Montana	Glyphosate	ND	0.54 (±0.10)	1.13 (±0.5)

All the values were calculated based on measured concentrations in tests with dilution series of field-applied soils. All units are in mg a.i./kg dry soil

ND not determined

Earthworm avoidance tests

No mortality was recorded in all avoidance tests performed. The dual control tests, in which the test boxes had the same concentration of pesticide in both sides, did not show any significant preference or aggregation to one side ($p > 0.05$) and mean distribution was 50 ± 10% in all replicates. After exposure to glyphosate more earthworms were found in the contaminated section (with a significant difference in the dilution of 50%), with the exception of the recommended field dose (Table 1). For glyphosate it was not possible to determine an AC_{50} (Table 2).

Earthworm reproduction tests

Mortality in the controls (≤10%), juvenile production per replicate (≥30) and coefficient of variation of reproduction (≤30%) met the validity criteria. No mortality was recorded in all reproduction tests performed. Exposure to glyphosate resulted in an increase in the number of juveniles in the contaminated test boxes when compared to the control soil, with significant differences in the 50% dilution (Table 3).

Collembolan avoidance tests

All tests performed with collembolans presented less than 20% mortality. The dual control tests did not show any significant preference or aggregation to one side ($p>0.05$) and mean distribution was 50 ± 10% in all replicates. Highest avoidance behaviour (48%) upon exposure to glyphosate was seen at the highest concentration, but this effect was not statistically significant (Table 1).

For glyphosate the AC_{50} was higher than 0.82 mg/kg dry soil with 48% avoidance at that concentration (Tables 2, 3).

Table 1. Percentage of avoidance and standard error calculated for the earthworm *Eisenia andrei* and collembolan *Folsomia candida* upon exposure to dilutions of contaminated soils treated with Montana®.

Treatment (%)	*Eisenia andrei* Avoidance (%)	*Folsomia candida* Avoidance (%)
M25	−16 (±1.9)	14 (±2.2)
M50	−52 (±0.9)*	6 (±1.7)
M75	−32 (±1.9)	28 (±0.8)
M100	16 (±3.4)	48 (±0.8)

* indicates statistically significant differences (Fischer's Exact Test); M is for MONTANA.

Table 3. Observed values and standard error of the reproduction success of the earthworm *Eisenia andrei* and adult survival and reproduction success of the collembolan *Folsomia candida* upon exposure to dilutions of contaminated soils treated with Montana®.

Treatment (%)	*Eisenia andrei*	*Folsomia candida*	
	Number of juveniles	Number of adults	Number of juveniles
Control M	50 (±5.1)	9.6 (±0.5)	405 (±80.6)
M25	76.6 (±9.1)	9.2 (±1.1)	430 (±68.9)
M50	91 (±33.9)*	6.6 (±0.8)*	294 (±87.5)
M75	83 (±16.8)	7 (±0.9)*	140 (±50.7)*
M100	48 (±9.4)	5.8 (±0.7)*	85 (±28.7)*

Concentrations are given as % of contaminated field soil in mixtures with control soil. M is for Montana®.
No mortality was recorded in all reproduction tests with earthworms. * significant differences (Dunnett's method)

Collembolan reproduction tests

Mortality in the controls (≤20%), juvenile production per replicate (≥100) and coefficient of variation of reproduction (≤30%) met the validity criteria. When exposed to Montana®, only in the two highest concentrations a significant effect was observed (Table 3). The EC_{50} values for Montana® was 0.54 mg/kg dry soil (Table 2). For glyphosate the LC_{50} value (1.16 mg dry soil) was twice the respective EC_{50} value and higher than the highest concentration tested (Table 2).

Conclusion

Eisenia Andrei

With the exception of the recommended field dose, the majority of earthworms were found in the contaminated section with Montana®. Montana® also stimulated earthworm reproduction, since an increase in the number of juveniles was observed in all soil dilutions except for the recommended field dose. This stimulatory effect observed with sub-lethal concentrations of this herbicide could be linked with the attraction observed for glyphosate, hence not only the earthworms were attracted to the herbicide treated soil but their reproductive output increased in the presence of this product. The application of Montana® at labelled doses does not seem to impair this species from inhabiting the soil where this pesticide is applied, probably because the effects of herbicides on soil invertebrates are mainly indirect and result from the disturbance of the vegetation.

Folsomia candida

In all treatments made with Montana®, more collembolans were found on the control section of the test vessels, however the results were never statistically significant different compared to the control, and only at the recommended field dose approximately 50% of the earthworms avoided the treated soil. The present results seem to corroborate these previous studies about glyphosate impact on collembolan species, since the behavioural pattern of *F. candida* was not changed due to herbicide application. Exposure to Montana® resulted in a gradual decline in the number of adults surviving the test period and a decrease in the number of juveniles produced. The EC_{50} value was very similar to the value obtained in a previous work where a commercial formulation containing glyphosate was spiked on soil LUFA 2.2 (Santos et al. 2010).

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study is well described and performed according to ISO guidelines. Validity criteria were met, where relevant. Glyphosate-based formulation Montana® did not seem to affect either earthworms or collembolans in the recommended field dose. There were no endpoints presented in the paper, thus the study is considered supplementary only.
In addition, a glyphosate based formulation was tested which is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
3 commercial formulations containing the insecticides chlorpyrifos and endosulfan and the herbicide glyphosate were applied to a Mediterranean agricultural field. The soil was collected after spraying and dilution series were prepared with untreated soil to determine the impact of the pesticides on the avoidance behaviour and reproduction of the earthworm Eisenia andrei and the collembolan Folsomia candida.
Concentrations tested are relevant. Measured concentrations varied between 0.340 and 1.230 mg/kg dw with a geomean of 0.822 mg/kg dw (a.i. glyphosate). Such variability was attributed to cross-contamination i.e. drift from one plot to an other during application.
The study authors stated that "*Glyphosate did not seem to affect either earthworms or collembolans in the recommended field dose*". This was because LC50 for Folsomia (1.13 mg/kg soil) was above the recommended field dose. This statement is not agreed by RMS, besides EC50 determined for F. candida (0.54 mg/kg soil) was below the recommended field dose. The reliability of these LC50 and EC50 values cannot be assessed (no data presented). Furthermore, concentrations of 50% of field rate (soil dilution 50%, i.e. treated soil mixed with control soil at ratio 1:1) induced significant effects on number of adults of F. candida. Dilution of 75% of the field rated induced significant effects on the number of juveniles (F. candida).
The effects on F. candida are notably different than those obtained in regulatory study.
No NOEC was calculated by RMS (although this is in principle possible by considering the dilution of 25% for which no significant effect was observed) as the exposure is deemed not reliable enough to derive a robust endpoint (high variability between samples). This study may however indicate strong effects at concentration lower than those expected in realistic conditions of use.

However these results are of limited value for other formulations than Montana, as toxicity of the glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant system in the product.
No adverse effects on earthworms were noted.

The study is considered less relevant but supplementary (due to the different formulation tested) and reliable with restrictions.

B.9.4.2. Effects on non-target soil meso- and macrofauna (other than earthworms)

B.9.4.2.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.4.1/005 also referenced under CA 8.4.2.1/005 and CA 8.5/005
Report author	von Mérey, G. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil biota
Document No	DOI: 10.1002/etc.3438 E-ISSN: 1552-8618
Guidelines followed in study	OECD 222; OECD 226; OECD 232; OECD 216
Deviations from current test guideline	Earthworm cocoons were not counted, in accordance with OECD 222.
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability (RMS):	Yes/Reliable This publication actually corresponds to the regulatory studies summarized and already assessed by RMS

	CA 8.4.1/001 [redacted] 2009 Glyphosate IPA salt Eisenia andrei CA 8.4.1/003 [redacted], 2003 AMPA Eisenia fetida CA 8.4.1/001 [redacted] 2010 Glyphosate IPA salt Folsomia candida CA 8.4.1/002 [redacted], 2009 Glyphosate IPA salt Hypoaspis aculeifer CA 8.4.2.1/003 [redacted] 2010 AMPA Folsomia candida CA 8.4.2.1/004 [redacted] 2010 AMPA Hypoaspis aculeifer CA 8.5/001 [redacted], 2014 Glyphosate Nitrogen transformation CA 8.5/004 [redacted] 2010 AMPA Nitrogen transformation Therefore, this publication was not assessed by RMS.

See B.9.4.1 above.

B.9.4.2.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 8.4, CP 10.4.2.2
Report author	Santos M. J. G. et al.
Report year	2012
Report title	Pesticide application to agricultural fields: effects on the reproduction and avoidance behaviour of Folsomia candida and Eisenia andrei.
Document Source	Ecotoxicology (2012), Vol. 21, No. 8, pp. 2113-2122
Guidelines followed in study	ISO 17512-2, ISO 11268-2 and ISO 11267 guidelines for laboratory phase
Deviations from current test guideline	No major deviations
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion by RMS:
See B.9.4.1.2

B.9.5. EFFECTS ON SOIL NITROGEN TRANSFORMATION

B.9.5.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.5/005, also referenced under CA 8.4.2.1/005
Report author	von Mérey, G. *et al.*
Report year	2016
Report title	Glyphosate and aminomethylphosphonic acid chronic risk assessment for soil biota
Document No	DOI: 10.1002/etc.3438 E-ISSN: 1552-8618
Guidelines followed in study	OECD 222; OECD 226; OECD 232; OECD 216
Deviations from current test guideline	Earthworm cocoons were not counted, in accordance with OECD 222.
GLP/Officially recognised testing facilities	No, not applicable
Acceptability/Reliability (RMS):	**Assessment and conclusion by RMS:** This publication actually corresponds to the regulatory studies summarized and already assessed by RMS CA 8.4.1/001 [redacted], 2009 Glyphosate IPA salt Eisenia andrei CA 8.4.1/003 [redacted] 2003 AMPA Eisenia fetida CA 8.4.1/001 [redacted], 2010 Glyphosate IPA salt Folsomia candida CA 8.4.1/002 [redacted] 2009 Glyphosate IPA salt Hypoaspis aculeifer CA 8.4.2.1/003 [redacted] 2010 AMPA Folsomia candida CA 8.4.2.1/004 [redacted] 2010 AMPA Hypoaspis aculeifer CA 8.5/001 [redacted], 2014 Glyphosate Nitrogen transformation CA 8.5/004 [redacted],, 2010 AMPA Nitrogen transformation Therefore, this publication was not assessed by RMS.

See under B.9.4.1

Data point:	CA 8.4.2
Report author	Rose M. T. et al.
Report year	2018
Report title	Minor effects of herbicides on microbial activity in agricultural soils are detected by N-transformation but not enzyme activity assays.
Document Source	European journal of soil biology (2018), Vol. 87, pp. 72-79
Guidelines followed in study	OECD Guideline 216 with minor modifications
Deviations from current test guideline	Not specified
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

The aim of this study was to quantify the effect of the herbicide glyphosate (acid) (and six other substances), on microbial activities and N-transformation in 5 different Australian broadacre (cereal) cropping soils. Glyphosate was applied at a recommended and 5 times recommended rate. Mineral N-levels were monitored over a 28 day period. Experiments were established as per OECD Guideline 216 with minor modifications.
No significant effects on NO_3^- formation and NH_4^+ levels were determined for the applications rates of glyphosate at 1 kg/ha (corresponding to 1.33 mg/kg) and 5 kg/ha (corresponding to 6.67 mg/kg) compared to the control.

Materials and methods

Test item
Technical grade (> 96% purity) glyphosate acid.
The test application rates were: 1 kg/ha (corresponding to 1.33 mg/kg) and 5 kg/ha (corresponding to 6.67 mg/kg).
Tebuconazole was used as positive control at the label rate of 0.125 kg/ha (corresponding to 0.166 mg/kg) and the 5 × label rate of 0.625 kg/ha (corresponding to 0.83 mg/kg).

Test soil
Topsoil (0–200 mm) was collected from 5 different fields with a previous history of grain cropping. Around 1000 kg of each soil was collected 6–9 months prior to the experimental setup and transported to the lab where they were stored as received in the dark at room temperature (20–25 °C). Soil samples were collected during the dry summer fallow period, such that all soils were <25% water holding capacity at sampling and during storage of bulk soil. Between 4 and 6 weeks prior to experimental setup, subsamples (approximately 100 kg) of each soil were air-dried in an air-conditioned glasshouse for 1 week (25 °C), sieved (< 2 mm) and homogenized, with a smaller portion (1 kg) further homogenized, analysed for a range of physicochemical properties, and used to establish sample treatments.
A description of the soils used is shown in the table below.

Table 1: Physicochemical properties residues of the soils.

Property	Unit	Tenosol	Chromosol	Sodosol	Vertosol	Calcarosol
FAO Classification[a]	–	Arenosol	Podzol	Solonetz	Vertisol	Calcisol
Location (GPS)		−30.84, 116.72	34.41, 147.52	−35.34, 145.66	−28.21, 152.10	−33.24, 134.72
Sand	%	85	48	35	42	66
Silt	%	3	27	19	15	11.5
Clay	%	12	25	36	43	22.5
pH (CaCl2)	pH	5	4.7	4.3	5.7	7.9
pH (Water)	pH	5.8	5.2	5.1	6.7	8.6
CEC	cmol(+)/kg	1.7	11	14	42	27
EC	dS/m	0.036	0.34	0.12	0.12	0.25
Total Organic Carbon	%	0.3	2.8	1.3	2	3
Total Nitrogen	%	0.03	0.25	0.13	0.15	0.16
KCl Extractable Ammonium	mg/kg	< 0.3	31	4.6	0.84	5.1
KCl Extractable Nitrate	mg/kg	5.9	140	39	35	9.7
Sulfur (KCl40)	mg/kg	5.2	30	9.9	8.4	12
Colwell Phosphorus	mg/kg	5.8	150	96	83	39
Phosphorus Buffer Index $_{+Col\ P}$	L/kg	15	60	220	110	210
Chloride	mg/kg	8.9	49	28	7.1	130
Boron	mg/kg	0.71	1.1	1.3	1.4	2.8
Exchangeable Cations						
Aluminium	cmol(+)/kg	0.15	0.15	0.51	< 0.1	< 0.1
Calcium	cmol(+)/kg	0.98	6.8	6.8	23	21
Potassium	cmol(+)/kg	0.15	2.1	0.85	0.99	1.7
Magnesium	cmol(+)/kg	0.33	1.5	6	17	3.9
Sodium	cmol(+)/kg	0.071	0.14	0.36	1.5	0.51

[a] IUSS Working Group WRB. 2015. World Reference Base for Soil Resources 2014, update 2015 International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome.

Prior to establishing treatments, soils were also analysed for residues of the herbicides. The only herbicide detected was glyphosate in the Chromosol (at 0.61 mg/kg) and the Sodosol (at 1.80 mg/kg).
Total C and N (Dumas combustion) were measured using an Elementar vario MAX CN analyser at 900 °C and oxygen flow rate of 125 mL/min. Colwell (bicarbonate-extractable) P and P buffer index were determined by methods 9B2 and 9I2b, respectively. Sulfur was extracted by 0.25M KCl at 40 °C at 3 h (method 10D1). Exchangeable cations were quantified after extraction with 1M NH_4OAc (method 15E1) and analysis with a Varian 720-EC ICP-OES, Inductively Coupled Plasma, Optical Emission Spectrometer (ICP-OES).

Test design
For each treatment, 4 replicates of 5 g of air-dried soils were prepared. Samples were pre-incubated at 25 % of maximum water holding capacity (WHC) for 7 days, under constant temperature of 20 °C, to restore microbial activity to air-dried soil.
Glyphosate was applied at the label rate of 1 kg/ha (corresponding to 1.33 mg/kg) and at the 5× label rate of 5 kg/ha (corresponding to 6.67 mg/kg). The dose required (mg/kg) was calculated according to an average soil bulk density of 1.5 g/cm3 and an assumed depth of incorporation of 50 mm.
Glyphosate was dissolved in water and then the soil was spiked with a sufficient volume of the diluted herbicide to bring the soil moisture to 60 % of the maximum water holding capacity (WHC) and the herbicide concentration to the required level.
Two sets of negative controls, 4 replicates each, were established, consisting of unspiked soil with an equivalent amount of water or sand to match water-soluble or water-insoluble agrochemical treatments, respectively.
Soils were destructively sampled at 7 days and 28 days after treatment application.

Enzyme activity and mineral N assay
This part is not reported by RMS as it is not relevant for the risk assessment.

Data analysis

The relative influence of soil type, measurement time and treatment on patterns of microbial activity was initially explored using non-metric multidimensional scaling (NMDS). This unconstrained ordination method separates dissimilar samples through numerical optimization to minimize a goodness-of-fit parameter (stress). NMDS was performed using the metaMDS function in the 'vegan' package in R 2.15.1. A Bray-Curtis dissimilarity index was calculated on transformed data (Wisconsin double standardisation) with the number of dimensions set to 2 and random starts applied to ensure a global solution (i.e. to overcome the possibility of solving for a local optimum).

Subsequently, the specific effects of herbicide treatment on N-transformation (NO_3^- formation rate and NH_4^+ concentration) were determined. Separate one-way ANOVAs were conducted for each soil type, at each measurement time, with herbicide treatment as the sole fixed factor in the model. A post-hoc Dunnett's test was used to identify treatments which were significantly different to the control group at P=0.05. Where assumptions of normality and homoscedasticity were not met, transformations were carried out and compared to results of untransformed data. Where similar statistical significance was obtained, results of the untransformed data were presented. The coefficient of variation of controls was calculated as the standard deviation of the endpoint measure (either NO_3^- formation rate, NH_4^+ concentration) at day 28 in the control group, divided by the average value of the control group at this time.

The NO_3^- formation rate data were presented as a table of percentage change relative to control values. However, because NH_4^+ is an intermediate in the nitrification pathway (with contributions from organic-N mineralisation and losses due to nitrification), data were presented as NH_4^+concentration, rather than rate of formation.

Data analysis was carried out on R 2.15.1

Results

No significant effects on NO_3^- formation were determined for both applications rates of glyphosate compared to the control. In the Vertosol, NO_3^-formation was retarded by the positive control tebuconazole at both application rates. Tebuconazole at label rates reduced NO_3^- formation in the Tenosol by 23 %. There were no significant effects on NO_3^- levels in the Calcarosol, Chromosol and Sodosol.

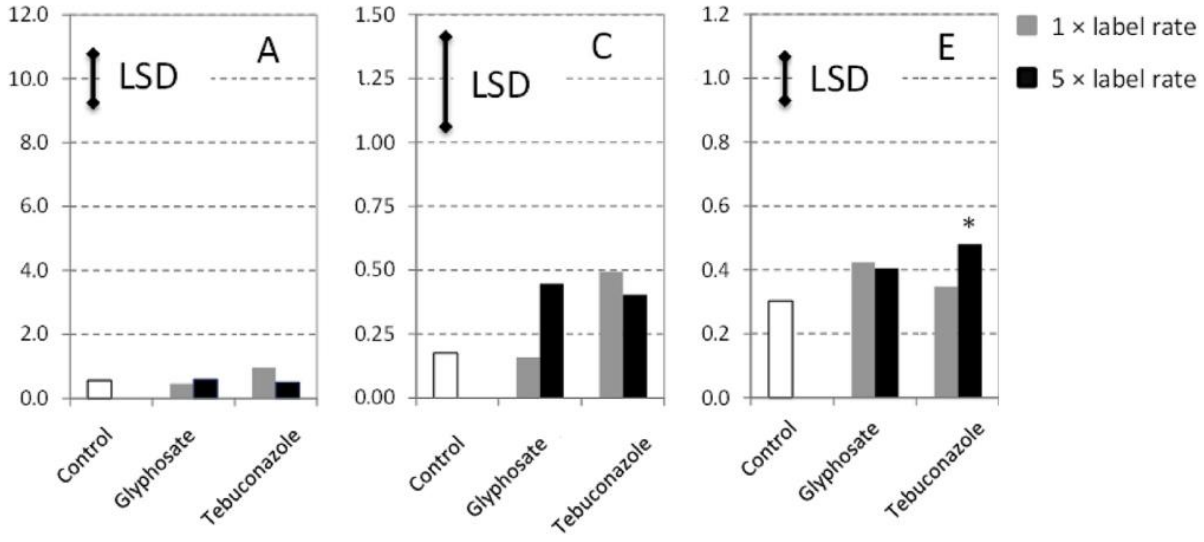
Table 2: Soil NO_3^- formation over 28 d following herbicide (or tebuconazole) application. Values are given as % of soil NO_3^- formation rate in controls (no herbicide).

Herbicide	Application Rate	Vertosol	Calcarosol	Chromosol	Sodosol	Tenosol
Glyphosate	1	90	92	102	95	91
	5	93	95	99	99	101
Tebuconazole	1	88	99	98	93	77
	5	89	113	91	94	89
Least significant difference *vs* control (Dunnett's test)		10	17	10	12	14
NO_3^--N formation rate in control treatment (mg kg^{-1} d^{-1}) [a]		1.17	1.34	2.39	1.84	0.12
Coefficient of variation of controls (%)		3.9	6.4	1.2	2.3	11.5

[a] Controls for water soluble herbicides (water) and non-water soluble herbicides (sand) were not significantly different for any soil ($P > 0.05$). Water control values are presented.

The effects on NH_4^+ levels after 28 days of application in the Chromosol, Sodosol and Calcarosol are presented below. No significant effects on NH_4^+ levels were determined for both applications rates of glyphosate compared to the control. The 5× label rate of the fungicide tebuconazole resulted in significant higher NH_4^+ levels in the Calcarosol.

Figure 2: Amount of NH_4^+ present at 28 d after treatment with glyphosate and the positive control tebuconazole in the Chromosol (A), Sodosol (C) and Calcarosol (E).



Conclusion

No significant effects on NO_3^- formation and NH_4^+ levels were determined for the applications rates of glyphosate at 1 kg/ha (corresponding to 1.33 mg/kg) and 5 kg/ha (corresponding to 6.67 mg/kg) compared to the control.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Non-EU soil but relevant endpoints demonstrating a lack of effects on soil microbial populations (n-trans) at field application rates.

Assessment and conclusion by RMS:

The aim of this study was to quantify the effect of the herbicide glyphosate (acid) (and six other substances), on microbial activities and N-transformation in 5 different Australian broadacre (cereal) cropping soils. Glyphosate was applied at a recommended and 5 times recommended rate. Mineral N-levels were monitored over a 28 day period. Experiments were established as per OECD Guideline 216 with minor modifications.

No significant effects on NO_3^- formation and NH_4^+ levels were determined for the applications rates of glyphosate at 1 kg/ha (corresponding to 1.33 mg/kg) and 5 kg/ha (corresponding to 6.67 mg/kg) compared to the control.
The soils used were not always as recommended in the OECD Guideline 216 (sand content, pH, total carbon content). RMS cannot ensure that adsorption of the test chemical was sufficiently minimized and its availability to the microflora sufficiently high.
Enzyme activity is not relatable to the risk assessment was not considered as a relevant parameter.

The study is considered relevant and reliable with restrictions (for soils characteristics).

B.9.5.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Newman M. *et al*.
Report year	2016
Report title	Glyphosate effects on soil rhizosphere-associated bacterial communities
Document No	The Science of the Total Environment, (2016) Vol. 543, No. Pt A, pp. 155-60
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by title/abstract

Full summary of the study according to OECD format

Glyphosate is one of the most widely used herbicides in agriculture with predictions that 1.35 million metric tons will be used annually by 2017. With the advent of glyphosate tolerant (GT) cropping more than 10 years ago, there is now concern for non-target effects on soil microbial communities that has potential to negatively affect soil functions, plant health, and crop productivity. Although extensive research has been done on short-term response to glyphosate, relatively little information is available on long-term effects. Therefore, the overall objective was to investigate shifts in the rhizosphere bacterial community following long-term glyphosate application on GT corn and soybean in the greenhouse. In this study, rhizosphere soil was sampled from rhizoboxes following 4 growth periods, and bacterial community composition was compared between glyphosate treated and untreated rhizospheres using next-generation barcoded sequencing. In the presence or absence of glyphosate, corn and soybean rhizospheres were dominated by members of the phyla *Proteobacteria*, *Acidobacteria*, and *Actinobacteria*. *Proteobacteria* (particularly *gammaproteobacteria*) increased in relative abundance for both crops following glyphosate exposure, and the relative abundance of *Acidobacteria* decreased in response to glyphosate exposure. Given that some members of the *Acidobacteria* are involved in biogeochemical processes, a decrease in their abundance could lead to significant changes in nutrient status of the rhizosphere. These results also highlight the need for applying culture-independent approaches in studying the effects of pesticides on the soil and rhizosphere microbial community.

Materials and methods

Greenhouse study

The soil used for the study was a Blount silt loam (fine, illitic mesic Aeric Epiaqualf). Soil pH was 6.95, and soil total C was 1.47%. Soil texture was 11% sand, 48% silt, and 41% clay. Typical Blount soil clay mineralogy is characterized by illite, hydroxyl-interlayered vermiculite, kaolinite, and quartz (Dontsova and Norton, 2002). Soil was collected in 2-cm increments to a depth of 39 cm, with 37 cm from the A horizon and the remaining 2 cm from the O horizon, from soil pits at a farm undergoing organic management in Delaware County, OH. This field site was previously under rotation of alfalfa–orchard grass–corn, oats–alfalfa–orchard grass, spelt–timothy–clover, and timothy–clover. The soil had never been exposed to glyphosate. Once collected, soil was stored in sealed plastic bags returned to the lab on ice and placed in rhizoboxes starting with the 38–39-cm increment, using ~62 g of soil per cm fill height. The soil was evenly distributed in the rhizobox and compacted to a bulk density of 1.3 g cm^{-3} and a total fresh soil weight within the rhizobox of 2500 g. A total of eight rhizoboxes were constructed as described

by Bott et al. (2008). Four rhizoboxes were planted for each of two crops, corn and soybean. Two rhizoboxes per crop were treated with glyphosate (Roundup PowerMax, Monsanto Company, MO, USA; active ingredient: glyphosate, N-(phosphonomethyl) glycine, in the form of its potassium salt), and two rhizoboxes served as untreated plant controls. These eight rhizoboxes were part of a larger ongoing research project that utilized all available rhizoboxes, leading to two rhizoboxes per treatment combination in this study. Plants were grown in eight growth periods over three years, with each growth period lasting 58 days. Plants were fertilized twice per growth period by applying 25 mL of fertilizer solution per rhizobox. Fertilizer solution was prepared by dissolving 3.745 g of Peters® 20/20/20 Professional fertilizer per liter, equaling 0.749 mg N, 0.749 mg P, and 0.749 mg K mL^{-1} of fertilizer solution. Fertilizer trace element concentrations were magnesium (0.019 mg mL^{-1}), boron (0.749 μg mL^{-1}), copper (0.002 mg mL^{-1}), iron (0.004 mg mL^{-1}), manganese (0.002 mg mL^{-1}), molybdenum (0.019 μg mL^{-1}), and zinc (0.002 mg mL^{-1}). The fertilizer was applied on days 30 and 50. The schedule for each period is outlined in Table 1. On day 1, before planting, all rhizoboxes were sprayed with glyphosate except for the controls. Glyphosate was applied at the recommended field rate (300.79 mL ha^{-1}). Corn and soybean seedlings germinated on cotton tissue were transplanted into rhizoboxes (2 plants/box) on day 10. Roundup Ready corn (Zea mays; DeKalb hybrid seed brand DKC62-54 (VT3)) and soybean (Glycine max; OX 20-8 RR) were used. Growth stages were estimated using the shortest periods given in the Ontario Agronomy Guide (Baute et al., 2002) for corn and soybean. On days 30 and 51 (when plants reached the V–5 and V–7 growth stages, respectively), glyphosate was applied on plant leaves using a cell spreader. Soil rhizosphere samples were collected on days 31, 37, 52, and 58. This schedule was then repeated for a total of eight growth periods. The rhizosphere soil samples used in this study were collected on day 58 of the fourth growth period.

Table 1
Schedule of events per growth period.

Day	Event
1	Glyphosate burn down spray
10	Corn and soybean planted
30	Glyphosate spray[a]
31	Collection of rhizosphere soil samples
37	Collection of rhizosphere soil samples
51	Glyphosate spray[b]
52	Collection of rhizosphere soil samples
58	Collection of rhizosphere soil samples

[a] Exact application at V3–V5 growth stages.
[b] Exact application at V6–V7 growth stages.

Sample collection and DNA extraction
Samples for this study were collected in the fourth growth period for corn and soybean. For the collection of rhizosphere soil samples, rhizoboxes were placed horizontally on the lab bench and clamps and the top acrylic plate were removed. Three 5-g subsamples of soil were collected using a spatula to recover soil within a 1-mm vicinity of the primary and lateral roots, avoiding the areas around the root tips and stored at −80 °C until further processing. These subsamples were processed separately, and the resulting sequence data was combined to account for variability in bacterial community composition within the rhizosphere. DNA was extracted from 500 mg of each soil rhizosphere sample using the UltraClean MicrobialDNA Isolation Kit (MoBio Laboratories, CA, USA) and eluted in 50 μl. DNA extracts were quantified using a Qubit Fluorometer and the dsDNA HS Assay kit (Life Technologies, CA, USA).

Sequencing library construction
PCR primers (515F/806R) designed by Caporaso et al. (Caporaso et al., 2012) were used to amplify the bacterial V4 hypervariable region of the 16S rRNA gene. Each primer contained the sequence adapter regions used by Caporaso et al. (Caporaso et al., 2012), and the reverse PCR primers contain a 12-base Golay barcode. Three sequencing primers were designed based on those of Caporaso et al. (Caporaso et al., 2012) to yield the 5′ read, the 3′ read, and the index read. See Table 2 for a description of the primers

used. PCR reagent mixes contained 12.5 μl KAPA HiFi HotStart Ready Mix (2×), 0.75 μl each of the forward and reverse primers (10 μMfinal concentration), 10 ng genomic DNA, and PCR water for a total reaction volume of 25 μl. The following touchdown PCR conditions were used: initial denaturation at 95 °C for 2 min followed by 32 cycles of denaturation at 98 °C for 20 s, annealing beginning at 61 °C and ending at 50 °C for 30 s, and extension at 72 °C for 30 s. The annealing temperature was lowered 1 °C every cycle until reaching 50 °C,which was used for the remaining cycles. Following this, a final extension of 72 °C for 10min was used. PCR products were purified by ethanol precipitation and verified on a 1% agarose gel. Positive amplicons were quantified using a Qubit Fluorometer and the dsDNA HS Assay kit (Life Technologies, CA, USA). Amplicons were pooled at equimolar concentrations, and the resulting pooled library was size-selected to remove smaller primer dimers. Since the 16S rRNA gene amplicon was approximately 420 bp, the E.Z.N.A. Size Select-IT Kit (Omega Bio-Tek, GA, USA) was used on the pooled bacterial 16S rRNA gene library, targeting 150–500 bp fragments. The library was quantified using a Qubit Fluorometer and dsDNA HS Assay kit (Life Technologies, CA, USA). The library was denatured with 0.2 N NaOH and diluted with pre-chilled HT1 buffer (Illumina, CA, USA) to a final concentration of 8 pM. The denatured and diluted library was spiked with 40% denatured PhiX and sequenced separately on an Illumina MiSeq (Illumina, CA, USA) using the sequencing primers mentioned above and a 300-cycle (2 × 150) MiSeq Reagent Kit v2 (Illumina, CA, USA).

Table 2
Primers used for amplification of bacterial 16S rRNA gene V4 hypervariable region.

Primers	Sequence (5′ to 3′)[a]
Forward (515F)	aatgatacggcgaccaccgagatctacacTATGGTAATTgtGTGCCAGCMGCCGCGGTAA
Reverse (806R)	caagcagaagacggcatacgagatNNNNNNNNNNNN[b]AGTCAGTCAGccGGAC TACHVGGGTWTCTAAT
Sequencing	
Read 1	TATGGTAATTgtGTGCCAGCMGCCGCGGTAA
Read 2	AGTCAGTCAGccGGACTACHVGGGTWTCTAAT
Index	ATTAGAWACCCBDGTAGTCCggCTGACTGACT

[a] Lowercase letters denote adapter sequences, underlined letters are pad regions, lowercase bold letters are linker regions, and uppercase letters are primer sequences specific to the V4/ITS1 regions.
[b] N's represent location of 12-base Golay barcode; see Caporaso et al. (2012) for a listing of the barcodes used.

Data analysis

Paired-end reads were assembled using PANDAseq (Bartram et al., 2011; Masella et al., 2012), and all downstream processing of sequences was completed using the QIIME pipeline v1.5.0 (Caporaso et al., 2010b). Assembled sequences were quality filtered using USEARCH v7 (Edgar, 2010), retaining only sequences >75 bases in length with expected errors >1.0. Chimeric sequences were identified and removed using USEARCH v6.1 (Edgar, 2010). Sequences were assigned to operational taxonomic units (OTUs) using open reference OTU picking in USEARCH v6.1 at a 97% similarity threshold. Reads were first clustered against the Greengenes 16S rRNA gene database (Aug 2013 release) (DeSantis et al., 2006), and then all remaining reads that did not cluster were clustered de novo. A representative sequence was chosen for each OTU based on which sequence was the most abundant for that given OTU. Taxonomy was assigned to the representative sequence of each OTU using uclust with the Greengenes 16S rRNA gene database (Aug 2013 release) (DeSantis et al., 2006). In addition, sequences identified as chloroplast following taxonomic assignment were removed. Reads were aligned using PyNAST (Caporaso et al., 2010a) against a reference alignment of the Greengenes core set (McDonald et al., 2011).

The generated OTU table was rarified to an even sampling depth (29,205 sequences per sample) using the *single_rarefaction.py* script in the QIIME pipeline, and this rarified OTU table was used to calculate alpha diversity metrics, including OTU abundance, Chao1 (Chao, 1984), Faith's phylogenetic diversity (Faith, 1992), and Shannon's index (Shannon, 1948). Alpha diversity metrics were compared between control and glyphosate-treated samples for each crop using the *compare_alpha_diversity.py* script in the QIIME pipeline which implements a nonparametric two-sample t-test with 999 Monte Carlo

permutations. Beta diversity metrics were also estimated using the rarified OTU table, including unweighted and weighted UniFrac distances (Lozupone and Knight, 2005). Weighted UniFrac distances were compared using a multiple response permutation procedure (MRPP)with 999 permutations. Principal coordinates analysiswas performed using the *beta_diversity_through_plots.py* script in the QIIME pipeline which uses the weighted UniFrac distances to generate plots and aid in visualization of the relationships among the various samples and treatments. All sequences obtained in this studywere submitted to the NCBI Sequence Read Archive (SRA) and are available under the study accession number PRJNA284763.

Results

Sequencing summary

Following assembly and quality filtering, a total of 505,391 bacterial 16S rRNA gene sequences were obtained with a range of 29,205 to 66,702 sequences per rhizobox and a mean of 49,249 sequences per rhizobox. All rarefaction curves tended to approach a plateau, indicating that the number of sequences obtained was sufficient to describe the bacterial diversity within these samples.

Rhizosphere bacterial community diversity

Alpha diversity estimates were similar between corn and soybean rhizospheres as well as among the control and glyphosate-treated samples (Table 3).

Table 3
Alpha diversity metrics for rhizosphere samples collected from control and glyphosate-treated rhizospheres of corn and soybean. Values represent mean ± 1SE.

	Observed OTUs	Chao1 richness estimate	Faith's phylogenetic diversity	Shannon's index
Corn				
Control	3814 ± 60	5872 ± 233	182.3 ± 3.1	10.2 ± 0.03
Glyphosate	4001 ± 86	6154 ± 111	189.7 ± 4.3	10.3 ± 0.05
Soybean				
Control	3849 ± 2	5946 ± 49	184.2 ± 0.2	10.2 ± 0.04
Glyphosate	3893 ± 101	5754 ± 210	184.2 ± 4.0	10.2 ± 0.08
Crop Effect[a]	0.656	0.428	0.604	0.91
Treatment Effect[a]	0.201	0.928	0.318	0.241

[a] Values represent p-values calculated using a nonparametric two-sample t-test with 999 Monte Carlo permutations.

Mean OTU abundance within corn and soybean control rhizospheres was 3814 and 3849, respectively. This increased slightly following glyphosate treatment to 4001 OTUs in corn and 3893 in soybean. Chao1 richness estimates increased in corn following glyphosate treatment (e.g. 5872 to 6154) but decreased in soybean (e.g. 5946 to 5754). Phylogenetic diversity observed within the corn rhizosphere was 182.29 in controls and 189.66 in glyphosate-treated samples, and there was no increase in phylogenetic diversity for soybean rhizosphere samples following glyphosate treatment. Shannon's diversity estimates were similar between control and glyphosate-treated rhizosphere samples with a mean Shannon's diversity estimate of 10.2. Weighted UniFrac distances showed that rhizosphere beta diversity varied by plant species ($p = 0.029$; $\alpha = 0.10$) but was fairly similar overall between control and glyphosate-treated samples ($p = 0.78$; $\alpha = 0.10$). Fig. 1 contains a PCoA plot of these results.

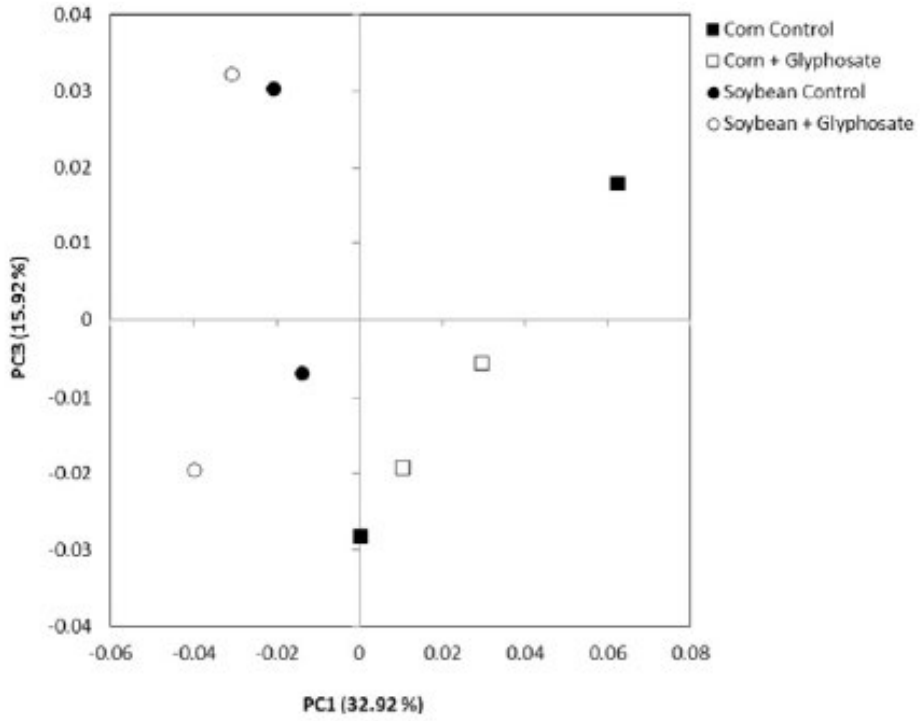


Fig. 1. PCoA plot based on weighted Unifrac distances generated for control (solid symbols) and glyphosate-treated (hollow symbols) rhizosphere bacterial communities of corn (squares) and soybean (circles) following four growth periods.

Rhizosphere bacterial community composition

Control samples and those receiving long-term glyphosate applications showed similarities in bacterial community composition at the phylum level. Control and treatment rhizosphere samples for both corn and soybean were dominated by members of the phyla *Proteobacteria*, *Acidobacteria*, and *Actinobacteria* (Fig. 2). The abundance of *Proteobacteria*-affiliated sequences increased in response to glyphosate treatment ($p = 0.096$). Corn rhizosphere samples showed an increase from an average of 22.9 ± 1.5% *Proteobacteria* sequences to 25.9 ± 0.9%. Soybean rhizosphere sample *Proteobacteria* sequences increased from an average of 25.4 ± 1.2% to 27.2 ± 0.2%. Within the sequences identified as belonging to the phylum *Proteobacteria*, no one bacterial class dominated for either corn or soybean. The *alphaproteobacteria*, *betaproteobacteria*, and *gammaproteobacteria* classes were present in controls samples for corn and soybean (ranging from approximately 5.2–8.3% relative abundance for each), as well as to a lesser extent the *deltaproteobacteria* (3.2% corn, 4.1% soybean).

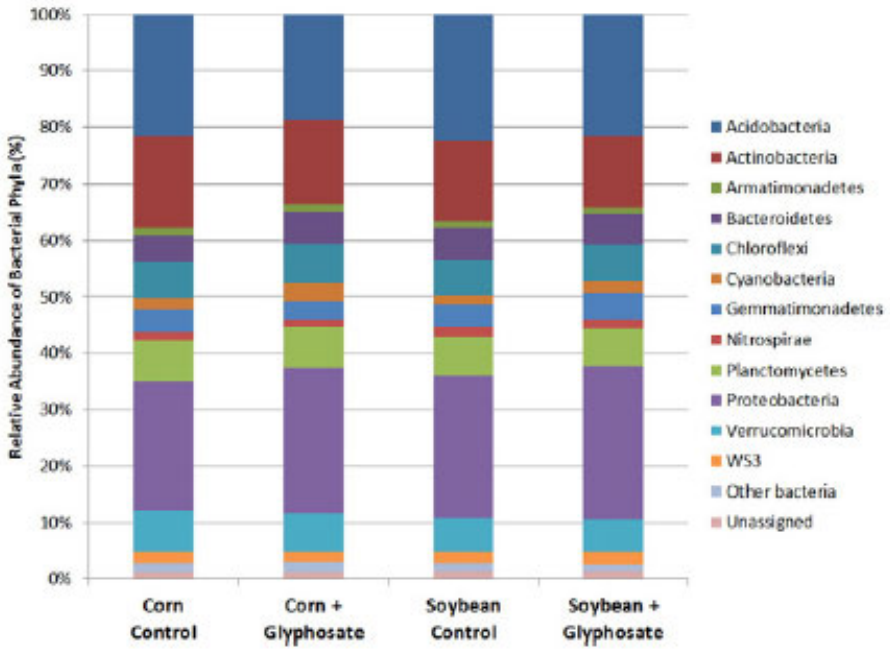


Fig. 2. Relative abundance of bacterial phyla present in control and glyphosate-treated rhizosphere bacterial communities of corn and soybean following four growth periods.

Following glyphosate treatment, all classes of *Proteobacteria* increased in relative abundance. *Gammaproteobacteria* sequences increased the most for both crops with an increase of 1.5% in corn and 0.7% in soybean. The majority of *gammaproteobacteria* sequences in control samples (2.6% corn, 3.2% soybean) were identified as belonging to the order *Xanthomonadales*, mainly from the families *Sinobacteraceae* and *Xanthomonadaceae*. Within the families *Sinobacteracea*e and *Xanthomonadaceae*, the majority of sequences matched reference sequences from unidentified genera. Other lower abundance genera present from these families included *Steroidobacter* within the family *Sinobacteraceae* and *Arenimonas*, *Dokdonella*, *Luteibacter*, *Lysobacter*, *Pseudoxanthomonas*, and *Thermomonas* within the family *Xanthomonadaceae*. The relative abundance of *Xanthomonadaceae* sequences increased for both crops following glyphosate treatment ($p = 0.081$). The response of *Sinobacteraceae* to glyphosate treatment varied with crop ($p = 0.003$). In corn, *Sinobacteraceae* relative abundance decreased (2.2% to 1.5%) following glyphosate treatment, but in soybean relative abundance of *Sinobacteraceae* increased (1.5% to 2.1%). In contrast, the relative abundance of members of the phylum *Acidobacteria* showed a decrease in response to glyphosate treatment ($p = 0.083$). In corn, the average relative abundance of *Acidobacteria* sequences decreased from 21.5 ± 1.1% in the control samples to 18.7 ± 0.8% in glyphosate-treated samples. For soybean there was also a decrease in the average relative abundance of *Acidobacteria* sequences from 22.3 ± 0.6% in control samples to 21.5 ± 0.3% in glyphosate-treated samples. The *Acidobacteria* subgroup 6, a dominant *Acidobacteria* subgroup in soils with few cultured representatives, made up the majority of *Acidobacteria* sequences for both corn (45.9%) and soybean (49.1%) and decreased in abundance following glyphosate treatment for corn, and to a lesser extent soybean. The average relative abundance of *Actinobacteria* also decreased following glyphosate treatment from 16.45% to 14.95% in corn and 14.35% to 12.6% in soybean ($p=0.445$).

Conclusion

The researchers examined the rhizosphere bacterial community composition response to glyphosate. Next-generation sequencing was used to examine the rhizosphere bacterial community. Relative abundance of Acidobacteria decreased in response to glyphosate exposure. Long-term glyphosate application could affect rhizosphere nutrient status.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by title/abstract: The article relates to long term cropping with treated crops and use of glyphosate. Therefore, not relatable to the glyphosate EU renewal from an ecotoxicological regulatory risk assessment perspective. Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). The tested formulation is not the representative formulation for the glyphosate EU renewal.
Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.

Assessment and conclusion by RMS:

This study investigates the rhizosphere bacterial community composition response to the application of glyphosate in formulation Roundup PowerMax. The formulation used is not well described (glyphosate content and surfactants are unknown). The study is then of limited relevance for the risk assessment of glyphosate. It is mentioned in the report that the application rate (300.79 mL/ha) corresponds to the recommended application rate. So the level of exposure is relevant.

Barcoded sequencing permitted detailed phylogenetic diversity analysis and was used to identify specific bacterial taxa shifts in the rhizosphere bacterial community in response to repeated glyphosate exposure on corn and soybeans.

Relative abundance of *Acidobacteria* decreased in response to glyphosate exposure. It is hypothesized by the study authors that long-term glyphosate application could affect rhizosphere nutrient status.

RMS considers the parameters investigated (rhizosphere bacterial community) as relevant as the effects of glyphosate may be masked by "functional redundancy" where overall soil functions are unaffected while microbial community composition is altered and key functions mediated by specific microbial populations are affected. Alterations to soil microbial community composition and subsequent changes in microbial diversity could potentially have pronounced long-term effects on soil quality and plant health.

The results of this study showed subtle alterations to rhizosphere bacterial community composition following the application of the herbicide glyphosate (in Roundup PowerMax). The largest shifts in relative abundance were observed for *Proteobacteria* (specifically *gammaproteobacteria*) and *Acidobacteria*. The increase in γ-Proteobacteria relative abundance for both corn and soybean rhizosphere samples was driven by increases in bacteria from the family *Xanthomonadaceae* following glyphosate treatment, suggesting that *Xanthomonadaceae* are adapted to and/or enriched by environments containing glyphosate.
Concomitantly, there were decreases in the relative abundance of *Acidobacteria*, particularly the *Acidobacteria* subgroup 6.

This study does not provide any information on whether this shift affects functional capability of the soil.

Overall, the study may be used to address biodiversity/indirect effects issues but is only considered as additional information due to the uncertainties around the test item.

Despite rather narrow uncertainty of the results, it is difficult to state on the reliability of the study as it does not follow a specific guideline. RMS notes that the number of replicates may also be considered too low (two rhizoboxes per treatment). No analytical verification was conducted.
The results from this study should be considered with caution. The study is considered reliable with restrictions.

The study is considered less relevant but supplementary and reliable with restrictions.

B.9.6. EFFECTS ON TERRESTRIAL NON-TARGET HIGHER PLANTS

B.9.6.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 8.6.2
Report author	Rogacz D. *et al.*
Report year	2020
Report title	Ecotoxicological effects of new C-substituted derivatives of *N*-phosphonomethylglycine (glyphosate) and their preliminary evaluation towards herbicidal application in agriculture.
Document No	Ecotoxicology and environmental safety, (2020) Vol. 194, Art. No. 110331.
Guidelines followed in study	OECD 208 (for NTTPs)
Deviations from current test guideline	No deviation indicated
GLP/Officially recognised testing facilities	No
Acceptability/Reliability:	supportive

Full summary of the study according to OECD format

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE 208 methodology (OECD 2006).

The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

Materials and methods

Glyphosate (≥ 98.0 %) was purchased from Aldrich, Poznań, Poland.

Plant growth test
The plant growth test of glyphosate was performed in laboratory conditions following the OECD 208 (2006).
According to the OECD 208 standard, the plant growth test was carried out in sandy soil of the following parameters: granulometric composition of soil: 77% sand, 16% dust and loam, organic carbon content of approx. 1.7%, pH (KCl) = 6.5.
Tests were carried out in polypropylene pots (diameter of 90 mm and capacity of 300 cm^3), which were filled with the control soil or with the soil mixed with the tested compounds added at following concentrations: 100, 200, 400, 800 and 1000 mg/kg of soil dry weight (s.d.w.). Each concentration was done in triplicate (3 pots for oat, 3 pots for common radish).
Twenty seeds of each of the selected plant species were sown into the soil. Oat (*Avena sativa*) and radish (*Raphanus sativus*) were selected as the representing species of monocotyledonous and dicotyledonous plants respectively. Seeds of each species originated from the same source. Plants were grown for 14 days under controlled conditions: a constant humidity content at the level required for the plants (70% field water capacity), temperature (22 ± 2 °C), constant light intensity (7000 lux), were maintained in the system of 16h/day and 8 h/night.
The evaluation of phytotoxicity at applied concentrations was made by comparing the germination, dry weight of control plants sprouts (seedlings) with germination and of dry and fresh plants sprouts grown in the soil with an admixture of given amounts of the tested compounds. Inhibition of fresh mass, root and shoot of plants was measured as described in the work by Lewkowski et al. 2016a, 2016b; Rogacz et al. 2018, Lewkowski et al. 2017a, 2017b, Matusiak et al. 2013.

Pigment assay
Photosynthetic pigments content was determined according to a method reported by Oren et al. 1995. Fresh leaves (200 mg) were thoroughly homogenized in 20 mL of 80% acetone in a cooled mortar and centrifuged afterward. The content of chlorophyll A, chlorophyll B and carotenoids was calculated based on the absorbance at wavelength 470, 647 and 664 nm. The content of photosynthetic pigments was expressed in mg/g of dry weight.

Statistics
One-way analysis of variance (ANOVA) has been used for comparison of obtained results related to phytotoxicity. Tukey's test with $p < 0.05$ (STATISTICA 13.3) was used to determine the significance of reported differences. The data presented are expressed as the mean with standard deviation obtained from 3 measurement replicates.

Results

Seedling emergence and growth test on oat and radish
The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure to glyphosate are summarized in the table below.

Table 1: Effects of glyphosate on the shoot height, the root length and the fresh matter of oat and radish seedlings plant compared to control (mean ± SD, n = 15).

Compound concentration [mg a.s./kg s.d.w.]	**Mean shoot height inhibition ± SD [%]**	**Mean root length inhibition ± SD [%]**	**Mean inhibition to fresh matter ± SD [%]**
	Oat		
100	7.2 ± 0.1	18.6 ± 1.1	10.7 ± 0.1
200	18.2 ± 0.4	25.1 ± 1.0	19.6 ± 0.4
400	53.4 ± 1.2	33.1 ± 0.6	43.8 ± 0.5
800	84.2 ± 2.0	41.2 ± 0.3	85.4 ± 0.7
1000	94.0 ± 0.9	90.4 ± 0.3	88.2 ± 0.1
	Radish		
100	7.3 ± 0.5	19.2 ± 0.3	11.1 ± 0.1
200	18.3 ± 0.7	26.7 ± 1.1	21.4 ± 0.1
400	57.1 ± 0.6	88.0 ± 0.8	60.7 ± 0.1
800	85.4 ± 0.3	91.8 ± 0.1	87.1 ± 0.1
1000	95.6 ± 0.1	94.2 ± 0.1	94.1 ± 0.1

Inhibition of shoots, roots and fresh matter in both plants (oat/radish) have been observed at all glyphosate concentrations tested (100, 200, 400, 800 and 1000 mg a.s./kg of soil dry weight).

Germination
The percentage germination of the tested plants treated with glyphosate is shown in the table below.

Table 2: Average changes (mean of three replicates) of germination of oat and radish treated with glyphosate.

Compound concentration [mg a.s./kg s.d.w.]	**Number of Emerged Seedlings**		**Germination [%]**	
	Oat	**Radish**	**Oat**	**Radish**
Control	20	19	100	100
100	19	17	98	88
200	19	16	98	86
400	18	15	93	77
800	18	13	92	70
1000	17	12	86	65
% Germination refers to number of emerged plants expressed as a percent of control plants.				

The higher the concentration of glyphosate in the soil, the stronger adverse effect was observed. At the highest concentration (1000 mg/kg), percentage germination was lower for radish (65%) when compared to oat (86%).

Dry matter
Dry matter of both treated plants oat/radish increased with growing concentration of glyphosate in the soil.

NOEC, LOEC and EC_{50} values

Changes of dry matter level caused by glyphosate are in accordance LOEC and NOEC values for oat and radish seedlings.

Calculated values of NOEC and LOEC of glyphosate for oat/radish were **100 and 200 mg a.s./kg of s.d.w.**, respectively.
The EC_{50} values of shoots was **373.7 mg a.s./kg s.d.w.** for oat, and **357.8 mg a.s./kg s.d.w.** for radish.
The EC_{50} values of roots was **269.3 mg a.s./kg s.d.w.** for radish and **556.9 mg a.s./kg s.d.w.** for oat.
The EC_{50} values of fresh weight was **333.2 mg a.s./kg s.d.w.** for radish and **418 mg a.s./kg s.d.w.** for oat.

Changes of pigment levels
The chlorophyll content measured in the radish/oat leaves decreased upon exposure to glyphosate.
Glyphosate caused accumulation of carotenoids in leaves of both tested plants.

Conclusion

In this paper, ecotoxicological and herbicidal effect of glyphosate was studied on monocotyledonous oat (*A. sativa*) and dicotyledonous radish (*R. sativus*) following the OECD/OCDE methodology (OECD 2006).

The growth inhibition values of shoot height, root length and fresh matter of both plants after exposure of glyphosate are presented. The EC_{50} values of shoots was 373.7 mg a.s./kg s.d.w. for oat, and 357.8 mg a.s./kg s.d.w. for radish. The EC_{50} values of roots was 269.3 mg a.s./kg s.d.w. for radish and 556.9 mg a.s./kg s.d.w. for oat. The EC_{50} values of fresh weight was 333.2 mg a.s./kg s.d.w. for radish and 418 mg a.s./kg s.d.w. for oat.

Assessment and conclusion

Assessment and conclusion by applicant:

The study investigated the effects of glyphosate on the seedling emergence and growth of non-target terrestrial plants (oat and radish) based on OECD 208 guideline. Plants were exposed to glyphosate mixed into sandy soil at 5 concentrations between 100 and 100 mg a.s./kg of soil dry weight with 3 replicates each. There were 20 seeds of each plant species per test concentration sown into the soil. Evaluations were based on fresh mass, root length and shoot height of plants after 14 days of exposure.

The test design was adequately described, but the application of the test item into the soil is not specified. The seedling emergence was acceptable as recommended in the guideline (100 and 95 % in the control for oat and radish, respectively). However, the phytotoxic effects and the survival of the control plants during the study is not reported.
Reliable endpoints for the risk assessment of NTTPs can be obtained for glyphosate: EC_{50} value of 373.7 mg a.s./kg s.d.w. for oat based on shoot height and an EC_{50} value of 357.8 mg a.s./kg s.d.w. for radish based on shoot height.

The article is classified as reliable for NTTPs.

Assessment and conclusion by RMS:

Ecotoxicological and herbicidal effect of newly synthesized N-[(phosphono)(aryl) methyl]glycines (C-substituted glyphosate derivatives) and pure glyphosate (N-phosphonomethylglycine) were investigated on oat and radish based on OECD 208 guideline.
Only findings on pure glyphosate are deemed relevant by RMS. Results on glyphosate derivatives are not considered further by RMS.

Shoot height
Glyphosate gave the EC50 value 373.7 mg/kg s.d.w. for oat shoots, and 357.8 mg/kg s.d.w. for radish shoots.
Effects of glyphosate were observed on oat (even at its lowest applied concentration 100 mg/kg of soil dry weight) with the growth inhibition reaching 7.2%. A two-fold increase of the glyphosate concentration in the soil resulted in growth inhibition at the level of 18.2%. The increasing concentration of glyphosate in the soil (400, 800 and 1000 mg/kg of soil dry weight) caused respectively 53.4, 84.2 and 94.0% reduction of oat shoots in relation to the control.
Glyphosate revealed to impact significantly on the length of the shoots of dicotyledonous radish. The obtained growth inhibition percentage values were comparable to the values obtained for oat for the same concentrations.

Root length
Glyphosate was almost twice more toxic to radish roots (EC50 = 269.3 mg/kg) than to oat roots (EC50 = 556.9 mg/kg).
Glyphosate in the soil (100, 200, 400, 800 and 1000 mg/kg of soil dry weight) caused respectively 18.6, 25.1, 33.1, 41.2 and 90.4% reduction of oat root length in relation to the control.
Glyphosate in the soil (100, 200, 400, 800 and 1000 mg/kg of soil dry weight) caused respectively 19.4, 26.7, 88.0, 91.8 and 94.2% reduction of radish root lenght in relation to the control.

Fresh mass
Radish: EC50 = 333.2 mg/kg, oat: EC50 = 418 mg/kg.
Glyphosate in the soil (100,200, 400, 800 and 1000 mg/kg of soil dry weight) caused respectively 10.7, 19.6, 43.8, 85.4 and 88.2% reduction of oat fresh matter in relation to the control.
Glyphosate in the soil (100,200, 400, 800 and 1000 mg/kg of soil dry weight) caused respectively 11.1, 21.4, 60.7, 87.1 and 94.1% reduction of radish fresh matter in relation to the control.

Glyphosate caused accumulation of carotenoids in leaves of both tested plants.

The test item was not described in the article (according to the article, this is described in another supplementary document, not provided to RMS). The summary made by the applicant indicated that test item was the active substance glyphosate.
No information on the study methodology and environmental conditions were reported in this article. These informations are reported in a supplementary document.

RMS notes that the concentrations tested are above those expected in real conditions of use.
RMS considers that critical validity criteria are lacking (as highlighted by the applicant).

RMS considered that the study is relevant for risk assessment purpose but of low reliability. Thus the study will be considered as supportive and use for weight of evidence.

B.9.6.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

No study belong to this category. However, datagap has been identified.

B.9.7. EFFECTS ON OTHER TERRESTRIAL ORGANISMS (FLORA AND FAUNA)

B.9.7.1. Published literature studies identified by RMS as relevant and reliable or reliable with restrictions

Data point:	CA 9
Report author	Colbach N. *et. al.*
Report year	2018
Report title	Landsharing vs landsparing: how to reconcile crop production and biodiversity? A simulation study focusing on weed impacts
Document No	Agriculture, Ecosystems and Environment (2018), Vol. 251, pp. 203-217
Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Not relevant by title/abstract

See Appendix to Volume 3 CP on literature data related to biodiversity.

Data point:	CA 9
Report author	Garcia-Ruiz E. *et al*.
Report year	2018
Report title	Weeds and ground-dwelling predators' response to two different weed management systems in glyphosate-tolerant cotton: a farm-scale study
Document No	PloS one, (2018) Vol. 13, No. 1, pp. e0191408
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by title/abstract

Full summary of the study according to OECD format

The use of glyphosate, as a post-emergence broad-spectrum herbicide in genetically modified glyphosate-tolerant (GT) cotton, supposes a big change in weed management programs with respect to a conventional regime. Thus, alterations in arable flora and arthropod fauna must be considered when evaluating their potential impacts. A 3-year farm-scale study was conducted in a 2-ha GT cotton crop, in southern Spain, to compare the effects of conventional and glyphosate herbicide regimes on weed abundance and diversity and their consequences for ground-dwelling predators. Surveys reveal that weed density was relatively low within all treatments with a few dominant species, with significantly higher weed densities and modifications of the floristic composition in glyphosate-treated plots that led to an increase in the abundance of *Portulaca oleracea* and to a reduction in plant diversity. The activity-density of the main predatory arthropod taxa (spiders, ground beetles, rove beetles and earwigs) varied among years, but no significant differences were obtained between conventional and glyphosate herbicide regimes. However, significant differences between treatments were obtained for ground beetles species richness and diversity, being higher under the glyphosate herbicide regime, and a positive correlation with weed density could be established for both parameters. The implications of these findings to weed control in GT cotton are discussed.

Materials and methods

Site description

The experimental site was a commercial cotton field located at Lebrija, in the province of Sevilla, Andalucia, southern Spain. This region is one of the traditional cotton producing areas, where is grown 98% of existing cotton in Spain. The field was cultivated with cotton for at least the two previous years and populations of weeds naturally occur in it. The soil at the site of study was of clayey nature with low sand content, alkaline and with low organic matter content. This site is under a typically Mediterranean climate, with mild and wet winters and autumns and long dry summers. The 30-year average annual rainfall is of 598 mm, mostly distributed from October to May and almost absent during the summer. Rainfall during the growing seasons (May-August) was 43, 9 and 39 mm in 2008, 2009 and 2010, respectively, while 30-year average for the corresponding period is 58 mm. Climatic data were obtained from "Lebrija I" weather station (36°58'40"N, 6°7'30"W, 25 m.a.s.l.).

Experimental design
The experiments were carried out within a 2-ha field sown with "Glytol" glyphosate-tolerant cotton event `GHB614' (Bayer CropScience) during the cotton growing seasons from 2008 to 2010. The trial was laid out as a randomized complete block design, with two treatments (weed-management regimes) and four blocks of two plots. The two treatments were randomly assigned to the two plots within each block and maintained for three years. Plot size was 60 m by 30 m.

Each year, the field was chisel-ploughed and then disked and field cultivated for seedbed preparation. Then cotton was sown in a weed-free and finely prepared seedbed at a depth of 3 cm using a precision seeder at 150,000 seeds ha^{-1} (20 kg ha^{-1}) in rows spaced at 95 cm. Cotton seed was supplied coated with the insecticide `Gaucho' (Imidacloprid). The insecticide Teflutrin (0.5% w/w) was also applied at sowing at 8 kg ha^{-1} as soil disinfectant for early-season insect control, a common practice in conventional cotton production. No other additional insecticides were applied in the site since the initiation of the study. Sowing dates for this study were 7th July 2008, 12th May 2009 and 19th May 2010. In 2008, the sowing date was postponed until the beginning of July because of the delay in the availability of GM cotton seed.
Herbicide regimes consisted of:

- glyphosate-only (G) treatment and
- conventional standard (C) treatment, which was used as control (Table 1 and Fig 1).

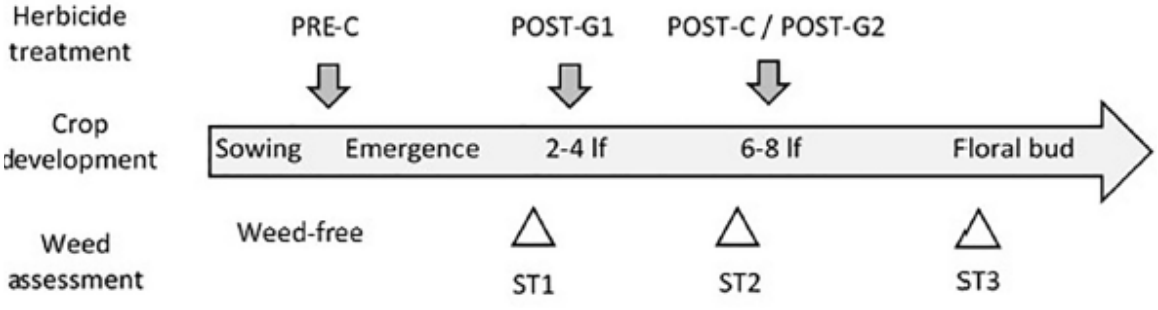
Table 1. Herbicide regimes (active ingredient, dose and application time) used in a genetically modified herbicide tolerant cotton.

Herbicide Treatment	Active ingredient (Commercial product)	Timing[a]	Dosage (kg a.i ha^{-1})	Application date (plant stage)[b]		
				2008	2009	2010
Conventional (C)	Fluometuron 25% + Terbutilazine 20.8% (Cottonex NeoPro)	PRE-C	0.88 + 0.73	July 8	May 13	May 20
	Clethodim 12% (Centurion Plus)	POST-C	0.18	na[#]	July 1 (8 lf)	June 28 (6 lf)
Glyphosate (G)	Glyphosate 36% (Roundup)	POST-G1	1.08	July 31 (4 lf)	June 16 (4 lf)	June 15 (3 lf)
	Glyphosate 36% (Roundup)	POST-G2	1.08	August 20 (8 lf)	July 2 (8 lf)	June 29 (6 lf)

[a] PRE: pre-emergence application (immediately after cotton was planted); POST: post-emergence application.
[b] Plant stage: cotton in 3rd (3 lf), 4th (4 lf), 6th (6 lf), and 8th leaf stage (8 lf).
[#] Herbicide not applied.

The G treatment included two applications of glyphosate in post-emergence (POST-G1 and POST-G2). The C program included a pre-emergence (PRE-C) herbicide application immediately after cotton was planted and a post-emergence (POST-C) application. The PRE-C herbicide possess residual activity has a broad-spectrum control of annual broadleaf and some grass weeds in their early stages of development. The POST-C graminicide was applied for annual grass weed control. In 2008, and because of the delayed sowing, there were no weeds in the conventional plots at the time of the post-emergence application, so POST-C treatment was not applied. All treatments were applied using a tractor-mounted sprayer with flat-fan nozzles calibrated to spray 180 L of solution per hectare.

Fig 1. Scheme of the herbicide treatments and weed assessments conducted in genetically modified herbicide tolerant cotton. All plots were weed-free at cotton sowing. PRE-C and POST-C emergence herbicides were applied only in conventionally (C) treated plots. POST-G1 and POST-G2 (glyphosate treatments) were applied only in glyphosate treated plots (G). Weed assessment was conducted in both C and G plots at three times: ST1) a few days before the application of POST-G1; ST2) 2-3 weeks after POST-G1; and ST3) 2-3 weeks after POST-C/POST-G2.



For a successful crop growth cotton, the same local agronomic practices, including tillage, were used for both weed management regimes. Cotton was irrigated by using an overhead sprinkler system every 2-3 weeks. The first irrigation was applied after sowing to ensure uniform germination, while next ones were applied always several days after each POST- herbicide application. The field was fertilized twice, with a basic dressing at a rate of 350 kg ha^{-1} NPK 7-10-6 before sowing and 350 kg ha^{-1} NPK 25-0-0 as top dressing. The conventional herbicide treatment was used to manage the weeds surrounding the experimental plots. Due to the fact that GM cotton (not approved for commercial use in the EU) was grown in an intensive cotton producing area, and that cross-pollination of neighbouring commercial non-GM cotton fields would result in GM-contaminated cotton seed, all the cotton plants were hand extracted just before reaching the flowering stage each year (5th September in 2008 and 29-31 July in 2009 and 2010). This GM plant material was destroyed in a nearby field by incorporating it into the soil with a disk harrow in compliance with Spanish regulations. Weeds remained in the experimental plots until the end of their cycle.

Weed assessments

Weed assessment was conducted in both C and G plots three times; ST1: a few days before the application of POST-G1; ST2: 2-3 weeks after POST-G1; and ST3: 2-3 weeks after POST-G2/POST-C (Fig 1). This time frame was chosen to get the best measure of the agronomic management impact on weeds, and to evaluate only those weeds that competed with the crop and would produce seeds for the next cycle.
Weed sampling was performed using 0.25 m^2 quadrats. Counts of individual weed plants identified to species (abundance) were made in thirty-four quadrats per plot (a total area of 8.5 m2). To estimate weed density (plants m^{-2}) and frequency, the quadrats were placed in a zigzag pattern along two diagonal transects (ten quadrats per transect) and in the four borders (three quadrats per short border and four per long border) of each plot, leaving a buffer of three meters to avoid edge effects. Frequency (*Fi*) was calculated as the percentage of quadrats in each plot in which the species *i* was present.

Arthropod assessments

Aboveground arthropods were monitored by using pitfall traps. Three traps were arranged diagonally across each plot, starting at least 10 m from the plot boundary to minimize potential edge effects. Each trap consisted of a plastic cup 12.5 cm in diameter and 12 cm deep with a plastic funnel fitted to the top, and both flush with the ground surface. An inner plastic 150 ml container half filled with a 1:1 mixture of water and ethanol was placed inside the plastic cup. The outer cup was punched to let the irrigation or rain water drain off. Traps were operative for 2 days every two weeks. A total of six sampling dates per year were performed from the end of May to mid-August in 2009 and 2010 and from the beginning of July to the end of September in 2008. All the individuals collected in the pitfall traps were taxonomically identified to the genus/species level in the main predatory groups and to at least the family level in the others. The number of arthropods captured in pitfall traps is a function not only of the density, but also of the activity of the sampled organisms and their behaviour on encountering a trap. Hence, the term ``activity-density" will be used to refer to population estimates obtained using pitfall traps.

Richness and diversity estimates

Species richness (S) and the Shannon-Wiener diversity index ($H' = -\Sigma\, pi \ln pi$, where *pi* is the relative abundance of each species) were used to detect changes in the community structure between herbicide management regimes for weed species and for the prevalent ground-dwelling insect predators collected (spiders, ground beetles and rove beetles).

Statistical analysis

The effects of herbicide management on weed density were analysed by a mixed-model ANOVA (year, herbicide management and sampling time of weeds as fixed factors and block as random). Differences in arthropods activity-density were tested by a mixed-model ANOVA (year and treatment as fixed

factors and block as random) with repeated measures (sampling date). All interactions were included in the models. Weed density (counts for all quadrats within a plot were pooled) data were log (x+0.5) transformed while arthropods activity-density (counts per pitfall trap) data were square root (x+0.5) transformed to stabilize variances. Weed and arthropod species richness and diversity data were pooled across sampling dates within plots and tested by two-way ANOVA (year and treatment) analyses. Pairwise comparisons were made by using the Student Newman-Keuls test (SNK) and the significance level of $P < 0.05$ was considered for all tests. All the analyses were carried out using the general linear model (GLM) procedure of IBM SPSS Statistics version 23.
Pearson's bivariate correlation analysis was performed to investigate relationships between annual arthropod variables (spider, ground beetle and rove beetle activity-density, richness and diversity and earwig activity-density) and total weed density across plots. Tests of statistical bilateral significance for the matrix of correlations (a total of 30 comparisons) were carried out considering the total data set (24 values for each variable: 3 years, 2 treatments and 4 blocks) and for each treatment separately (12 values: 3 years and 4 blocks).

Results

Weed community and density
Weed community in the present study consisted of a variable number of species with a relative low density and a few dominant species, as normally occurs in farming systems. Six annual weed species: *Abutilon theophrasti* Med, *Amaranthus retroflexus* L., *Chenopodium album* L., *Echinochloa colona* (L.) Link, *Portulaca oleracea* L. and *Solanum nigrum* L.; and two perennial: *Convolvulus arvensis* L. and *Malva sylvestris* L., were recorded during the study.

Table 2. Density and frequency of the main weed species (found at least in 5% of the quadrats in one of the conducted surveys) under conventional or glyphosate herbicide regime.

Weed species	Year	Weed Density[a] (Frequency)					
		Conventional			Glyphosate		
		ST1	ST2	ST3	ST1	ST2	ST3
Echinochloa colona	2008	0.97 ± 0.38 (0.15 ± 0.04)	1.65 ± 0.45 (0.30 ± 0.15)	1.74 ± 0.36 (0.31 ± 0.02)	7.94 ± 2.18 (0.54 ± 0.06)	0.56 ± 0.12 (0.13 ± 0.06)	0.03 ± 0.03 (0.01 ± 0.01)
	2009	9.56 ± 3.39 (0.44 ± 0.06)	10.97 ± 3.50 (0.59 ± 0.07)	0	1.59 ± 0.22 (0.31 ± 0.03)	0.18 ± 0.14 (0.04 ± 0.03)	0
	2010	0.85 ± 0.35 (0.15 ± 0.06)	1.76 ± 0.38 (0.35 ± 0.08)	0	0.56 ± 0.33 (0.10 ± 0.05)	0.03 ± 0.03 (0.01 ± 0.01)	0
Portulaca oleracea	2008	0.06 ± 0.06 (0.01 ± 0.01)	0.06 ± 0.03 (0.01 ± 0.05)	0.06 ± 0.06 (0.01 ± 0.01)	6.91 ± 2.82 (0.65 ± 0.13)	4.06 ± 1.63 (0.56 ± 0.28)	3.44 ± 1.24 (0.49 ± 0.14)
	2009	0.03 ± 0.03 (0.01 ± 0.01)	0.06 ± 0.03 (0.01 ± 0.01)	0.03 ± 0.03 (0.01 ± 0.01)	23.50 ± 4.37 (0.92 ± 0.05)	22.88 ± 5.11 (0.87 ± 0.09)	9.53 ± 1.99 (0.84 ± 0.11)
	2010	0.03 ± 0.03 (0.01 ± 0.01)	0.09 ± 0.03 (0.04 ± 0.01)	0.03 ± 0.03 (0.01 ± 0.01)	40.62 ± 12.3 (0.94 ± 0.06)	10.82 ± 4.70 (0.67 ± 0.09)	1.12 ± 0.39 (0.19 ± 0.07)
Solanum nigrum	2008	0	0	0	1.56 ± 0.52 (0.27 ± 0.10)	0	0
	2009	0	0	0	0	0	0
	2010	0.06 ± 0.06 (0.01 ± 0.01)	0.09 ± 0.03 (0.02 ± 0.01)	0.12 ± 0.05 (0.03 ± 0.01)	1.26 ± 0.60 (0.24 ± 0.10)	0	0

Weed assessment was conducted in both Conventional and Glyphosate treated plots: ST1) a few days before the application of POST-G1; ST2) 2–3 weeks after POST-G1; and ST3) 2–3 weeks after POST-C/POST-G2 (see Fig 1).
[a] Values represent the mean ± standard error (SE) of 4 plots per treatment (34 quadrats per plot).

Table 2 shows the density and frequency of *E. colona*, *P. oleracea*, and *S. nigrum*, the dominant weed species, found at least in 5% of the quadrats in one of the conducted surveys, under conventional or glyphosate herbicide regime. The other species were found to a much lesser extent: *A. retroflexus*, *C. album* and *M. sylvestris* were not detected in the C system and only occasionally (one sampling time) in the G system, while *A. theophrasti* and *C. arvensis* appeared occasionally in both herbicide systems. Both herbicide management regimes reduced the density of weeds, but the response of each species depended on the herbicide treatment. In plots managed with conventional herbicides, the application of the pre-emergence herbicide effectively reduced the emergence of weeds at ST1 and ST2, except for *E. colona* (Table 2). However, *E. colona* was completely controlled in 2009 and 2010 after the application of the post-emergence treatment. In plots managed with glyphosate, *E. colona*, *P. oleracea*, and *S. nigrum* were the most abundant weed species identified during the initial weed counts after the cotton

sowing (ST1) with up to 7.94, 6.91 and 1.56 plants m^{-2}, respectively (Table 2). Weed densities at ST3 show that almost all weeds except *P. oleracea* were suppressed by the two postemergence glyphosate treatments. In 2008 and 2009, the first glyphosate treatment was applied when cotton plants were at 4th leaf stage, but *P. oleracea* plants were then at the reproductive stage and survived the effect of the herbicide. When cotton received the second glyphosate application at the 8th leaf stage, the size of *P. oleracea* at that moment compromised the herbicide's efficacy: most of the plants could complete their life cycle and left a great seedbank for the following years. In 2010, glyphosate treatments were applied earlier (at 3rd and 6th leaf stage of the cotton) and most of *P. oleracea* plants were controlled. However, some *P. oleracea* plants were well developed even when cotton plants were at the cotyledon stage. Therefore, they could complete their cycle and leave seeds.
Weed density differed significantly between both herbicide management regimes, with significant lower total weed densities in the C than in the G system (Table 3).

Table 3. Total density of weeds growing under conventional or glyphosate herbicide regime.

Year	Treatment	Weeds Density (mean ± SE)[a]			Year (Y) [$F_{2,\ 28}$ (*P*)]	Treatment (T) [$F_{1,\ 28}$ (*P*)]	Sampling (ST) [$F_{2,\ 28}$ (*P*)]	Y x ST [$F_{4,\ 28}$ (*P*)]	T x ST [$F_{2,\ 28}$ (*P*)]
		ST1	ST2	ST3					
2008	Conventional	1.24 ± 0.60	1.62 ± 0.39	1.82 ± 0.44	28.45* (0.001)	29.46* (0.012)	64.47 (0.000)	92.30 (0.000)	62.47 (0.000)
	Glyphosate	17.0 ± 5.51	3.85 ± 1.01	3.68 ± 1.41					
2009	Conventional	9.59 ± 3.41	11.1 ± 3.49	0.03 ± 0.03					
	Glyphosate	25.1 ± 4.54	23.1 ± 5.20	9.53 ± 1.99					
2010	Conventional	1.06 ± 0.46	2.03 ± 0.56	0.24 ± 0.08					
	Glyphosate	42.7 ± 12.5	10.0 ± 3.94	0.91 ± 0.41					

Weed assessment was conducted in both Conventional and Glyphosate treated plots: ST1) a few days before the application of POST-G1; ST2) 2–3 weeks after POST-G1; and ST3) 2–3 weeks after POST-C/POST-G2 (see Fig 1). Means were compared by four-way ANOVA, and the F calculated for the factors year, treatment (herbicide regime), blocks and sampling times, as well as all associated interactions. Only significant factors and interactions are shown. Significant values ($P < 0.05$) are marked with *

[a] Average of 4 plots per treatment (34 quadrats per plot).

Significant differences on total weed density were also detected among years, being higher in 2009 than in 2008 and 2010 (SNK, P < 0.05, Table 3). The management done in 2008, in which the late sowing of the crop resulted in the lack of post-emergence application in the C system and in a delay of glyphosate application in the G system, allowed *E. colona* in conventional and *P. oleracea* in glyphosate to escape, increasing the seeds in the soil seed bank for the next year. Sampling time and the interactions `treatment by sampling time' and `year by sampling time' resulted also significant in accordance the herbicide applications (Table 3). Interestingly, the `year by treatment' interaction was not significant, indicating that differences between treatments were consistent over time.

Weed species richness and diversity

Weed species richness (S) was relatively low across the years in both herbicide management regimes, with mean number of species ranging from 1.5 to 3.1 species per year on glyphosate management and from 1.0 to 2.1 species per year on the conventional, with no significant differences between herbicide management regimes (Table 4). There were however significant differences among years (Table 4), with the lowest richness obtained in 2009 and the highest in 2010 (SNK, P < 0.05). `Year by treatment' interaction was not significant (Table 4).

Table 4. Species richness (S) and diversity index (H') of weeds in glyphosate tolerant cotton under conventional or glyphosate herbicide regimes.

	Year	Treatment (mean[a] ± SE)		Year (Y) [$F_{2,\ 18}$ (*P*)]	Treatment (T) [$F_{1,\ 18}$ (*P*)]	Y x T [$F_{2,\ 18}$ (*P*)]
		Conventional	Glyphosate			
Richness (S)	2008	1.75 ± 0.53	3.25 ± 0.64	4.60* (0.02)	1.29 (0.27)	1.67 (0.22)
	2009	1.00 ± 0.14	1.50 ± 0.10			
	2010	2.08 ± 0.63	2.17 ± 0.10			
Diversity (*H'*)	2008	0.22 ± 0.15	0.56 ± 0.05	3.31 (0.06)	3.73 (0.07)	4.66* (0.02)
	2009	0.02 ± 0.01	0.09 ± 0.01			
	2010	0.43 ± 0.16	0.10 ± 0.02			

Means were compared by two-way ANOVA, and the *F* calculated for the two factors (year and treatment) and their interaction. Significant values ($P < 0.05$) are marked with *

[a] Data were pooled across sampling dates within plots

There was a significant `year by treatment' interaction after the ANOVA on weed diversity data (Table 4), showing that weed diversity (Shannon-Wiener index, H') response was not consistent from 2008 to 2010, so differences between herbicide management regimes were reanalyzed within each year. There were no differences in weed diversity between glyphosate and conventionally-managed plots in the year 2008 ($F_{1,6}$ = 5.17, P = 0.063), whereas in 2009 weed diversity resulted significantly higher in G than in C system ($F_{1,6}$ = 57,09, P <0.001) and in 2010 resulted significantly lower in G than in C in 2010 ($F_{1,6}$ = 32.61, P = 0.002). This year weed presence was restricted to *P. oleracea* after the first treatment with glyphosate.

Arthropod taxa

A total of 3141 arthropods captured by pitfall traps were assessed during the three-year study (Table 5). Of these, the most abundant groups considered generalist predators were spiders (Araneae) (38%), followed by earwigs (Dermaptera) (32%), ground beetles (Coleoptera: Carabidae) (18%), rove beetles (Coleoptera: Staphylinidae) (9%) and centipedes (Chilopoda:Lithobiidae) (3%). Other groups found were crickets (Orthoptera: Gryllidae) of the genera Acheta and Gryllus (51% of the total), which feed on vegetable material or small prey; woodlices (Isopoda) of the family Porcellionidae (4%), mostly detritivores, and darkling beetles (Coleoptera: Tenebrionidae) (3%) which feed on both fresh and decaying vegetation.

The composition of the predatory groups varied among years. Thus, spiders' relative activity-density ranged from 19% of the total predators in 2008 to 65% in 2010. In 2008, 50% of the total predators were earwigs, but it was only 16% in 2010. Ground beetles ranged from 21% of the total predators in 2008 to 13% in 2010, and rove beetles between 10% in 2008 and 4% in 2010 (Table 5).

Table 5. Total number of the ground-dwelling arthropod groups captured in pitfall traps in glyphosate tolerant cotton plots.

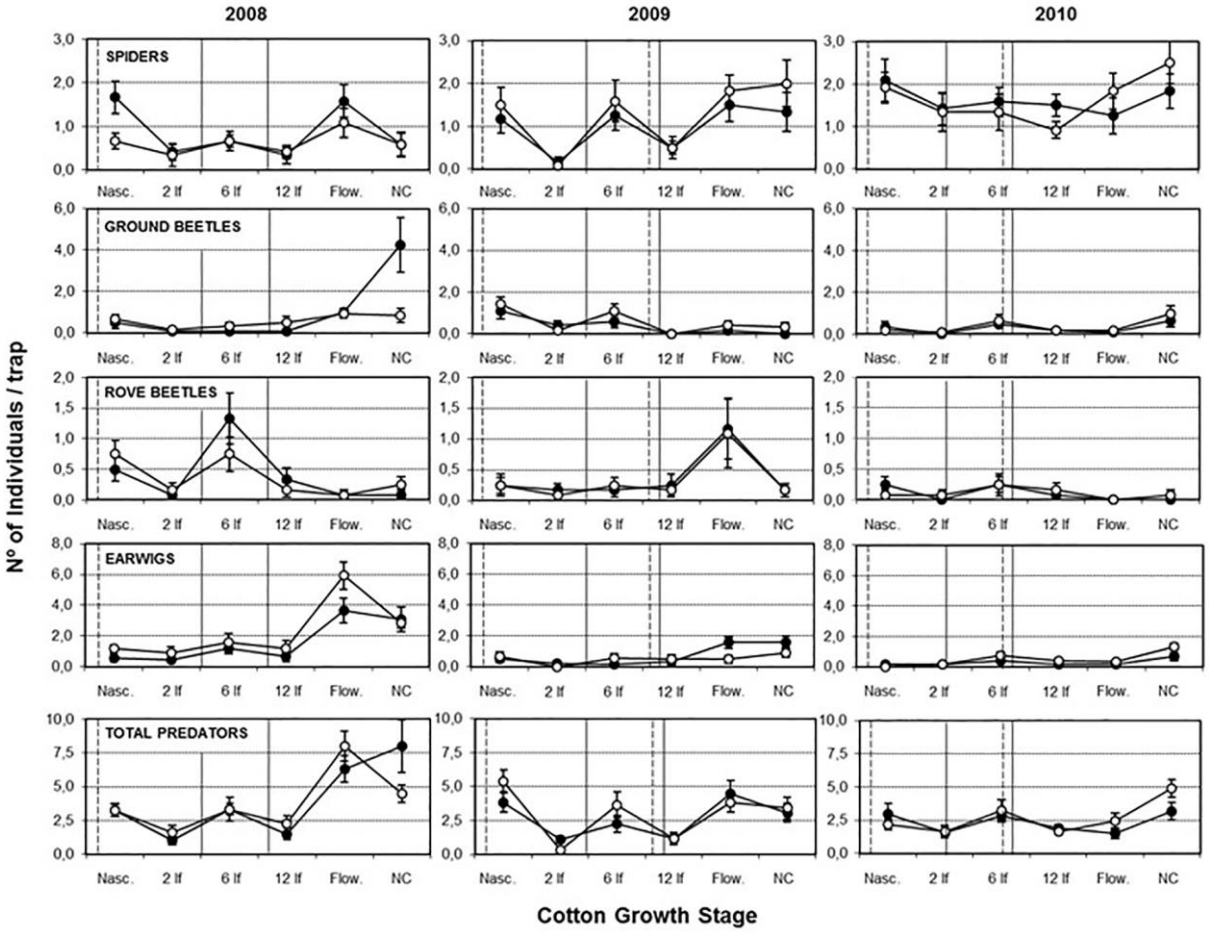
Arthropod group[a]	2008	2009	2010	Total
Crickets (ORT)	934	411	240	1585
Spiders (ARA)	108	161	233	502
Earwigs (DER)	278	91	57	426
Ground beetles (COL)	118	69	48	235
Woodlice (ISO)	41	18	91	150
Rove beetles (COL)	55	50	15	120
Darkling beetles (COL)	3	28	50	81
Centipedes (CHI)	2	35	5	42
Total	1539	863	739	3141

[a] In brackets: ORT, Orthoptera; ARA, Araneae; DER, Dermaptera; COL, Coleoptera; ISO, Isopoda; CHI, Chilopoda.

Activity-density predator patterns and community indicators

The activity-density patterns of the four main predatory groups (spiders, ground beetles, rove beetles and earwigs) oscillated throughout the cotton phenological stage (Fig 2). Only in the case of ground beetles a major peak was recorded in plots treated with conventional herbicides in the last sampling date of 2008 (with the crop already extracted), but not in the following years.

Fig 2. Activity-density patterns of the four main predatory groups: Spiders, ground beetles, rove beetles and earwigs. Mean number of individuals per trap (± SE) of the four most abundant ground-dwelling predator groups in glyphosate tolerant cotton under glyphosate (°) or conventional herbicides (●) regime. Sampling dates on the x-axis are cotton nascence (Nasc.), cotton in 2nd (2 lf), 6th (6 lf), and 12th (12 lf) leaf stage, cotton flowering (Flow.) and after cotton extraction (NC -No Crop-). The vertical lines indicate the glyphosate (–) and the conventional (- -) treatments.



The differences found in the activity-density values for these taxonomic groups strongly depended on the year, but no differences were observed between treatments (Table 6).

Table 6. Activity-density of ground-dwelling predatory groups in glyphosate tolerant cotton under conventional or glyphosate herbicide regimes.

Group	Year	Treatment (mean[a] ± SE)		Year (Y) [$F_{2, 48}$ (P)]	Treatment (T) [$F_{1, 48}$ (P)]	Y x T [$F_{2, 48}$ (P)]
		Conventional	Glyphosate			
Spiders	2008	0.88 ± 0.13	0.63 ± 0.10	29.09* (0.00)	0.00 (0.95)	2.43 (0.10)
	2009	0.99 ± 0.14	1.25 ± 0.17			
	2010	1.61 ± 0.16	1.64 ± 0.17			
Ground beetles	2008	1.00 ± 0.28	0.57 ± 0.10	10.33* (0.00)	0.47 (0.49)	3.14 (0.05)
	2009	0.38 ± 0.10	0.57 ± 0.11			
	2010	0.29 ± 0.08	0.38 ± 0.09			
Rove beetles	2008	0.40 ± 0.10	0.36 ± 0.07	15.16* (0.00)	0.35 (0.56)	0.18 (0.83)
	2009	0.36 ± 0.10	0.33 ± 0.11			
	2010	0.10 ± 0.04	0.11 ± 0.04			
Earwigs	2008	1.60 ± 0.26	2.26 ± 0.30	70.87* (0.00)	3.63 (0.06)	4.82* (0.01)
	2009	0.74 ± 0.13	0.53 ± 0.11			
	2010	0.29 ± 0.06	0.50 ± 0.09			

Means were compared by a mixed-model ANOVA (year and treatment as fixed factors and block as random) with repeated measures (sampling date) analyses. All factors' interactions were studied but only 'year by treatment' values are shown. Significant values ($P < 0.05$) are marked with *

[a] Means per pitfall trap

The `year by treatment' interaction was only significant for earwigs. Twenty-five species of spiders were found during the three years of sampling. Seven of them accounted for 80% of the total: *Robertus arundineti* O.P.-Cambridge (Theridiidae), *Diplocephalus graecus* O.P.-Cambridge (Linyphiidae), *Zelotes civicus* Simon (Gnaphosidae), *Hogna radiata* Latreille (Lycosidae), *Dictyna latens* Fabricius

(Dictynidae), *Agraecina lineata* Simon (Liocranidae) and *Thanatus vulgaris* Simon (Philodromidae) (Fig 3).

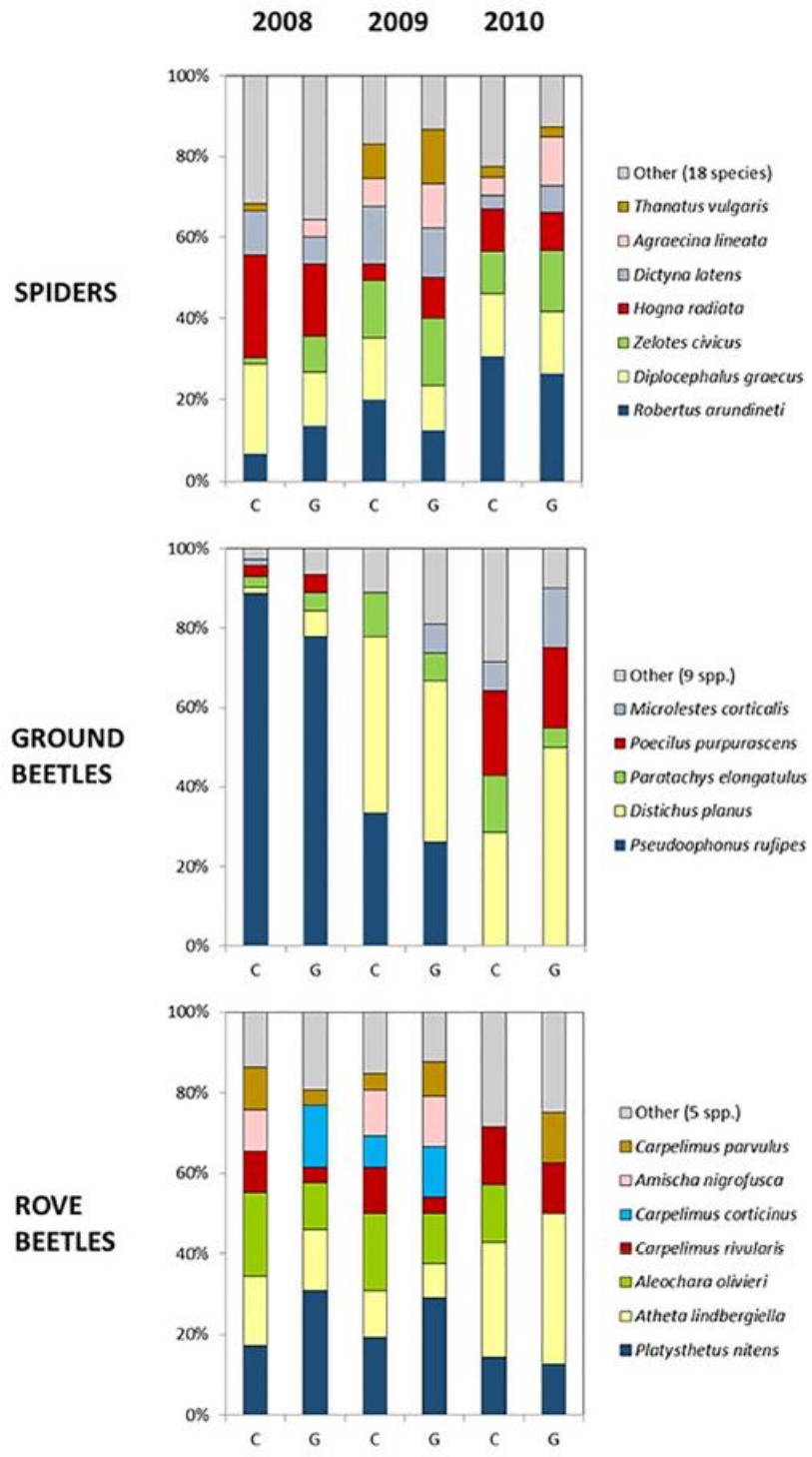


Fig 3. Species composition of spiders, ground beetles and rove beetles in glyphosate tolerant cotton under conventional (C) or glyphosate (G) herbicide regime. Other species: Ground Beetles (Carabidae): *Siagona europea*, *Calosoma maderae*, *Sphaerotachys lucasii*, *Scybalicus oblongiusculus*, *Paratachys bistriatus*, *Zuphium olens*,*Microlestes sp.*, *Bembidion vicinum*, *Ophonus ardosiacus*; Rove beetles (Staphylinidae): *Xantholinus vandalicus*, *Scopaeusmitratus*, *Achenium depressum*, *Anotylus nitidulus*, *Atheta laticollis*; Spiders (Araneae): *Pardosa sp*. (Lycosidae), *Aelurillus sp.* (Salticidae), *Agroeca sp*. (Liocranidae) and other 15 spider morphospecies that were not identified because of their low abundance (less than 10 captures in total).

Significant changes among years were recorded for the activity-density of *R. arundineti* ($F_{2, 48}$ = 25.94, P < 0.001), *Z. civicus* ($F_{2, 48}$ = 11.77, P < 0.001), *T. vulgaris* ($F_{2, 48}$ = 9.08, P < 0.001), *A. lineata* ($F_{2, 48}$ = 4.61, P = 0.015) and *D. graecus* ($F_{2, 48}$ = 3.54, P = 0.037). However, only *H. radiata* showed significant differences between treatments, the activity-density being higher under the glyphosate herbicide regime ($F_{1, 48}$ = 7.92, P = 0.007), and *A. lineata* for `year by treatment' interaction ($F_{2, 48}$ = 3.40, P = 0.042).

Table 7. Species richness (S) and diversity index H') of spiders, ground beetles and rove beetles in glyphosate tolerant cotton under conventional or glyphosate herbicide regimes.

	Year	Treatment (mean[a] ± SE)		Year (Y) [$F_{2, 18}$ (*P*)]	Treatment (T) [$F_{1, 18}$ (*P*)]	Y x T [$F_{2, 18}$ (*P*)]
		Conventional	Glyphosate			
Richness (*S*)						
Spiders	2008	7.0 ± 0.7	7.0 ± 2.0	3.23 (0.06)	0.01 (0.94)	0.22 (0.81)
	2009	9.0 ± 1.9	9.8 ± 1.4			
	2010	10.8 ± 0.5	9.8 ± 0.8			
Ground beetles	2008	3.0 ± 1.2	3.5 ± 0.5	0.21 (0.81)	4.64* (0.04)	0.79 (0.47)
	2009	2.5 ± 0.5	5.0 ± 0.6			
	2010	3.0 ± 0.9	4.3 ± 0.9			
Rove beetles	2008	5.3 ± 0.3	4.3 ± 0.9	5.15* (0.02)	0.25 (0.62)	0.13 (0.88)
	2009	4.5 ± 1.7	4.3 ± 1.3			
	2010	1.8 ± 0.5	1.8 ± 0.8			
Diversity (*H'*)						
Spiders	2008	1.76 ± 0.12	1.69 ± 0.27	2.34 (0.13)	0.23 (0.88)	0.12 (0.89)
	2009	1.98 ± 0.16	2.04 ± 0.12			
	2010	2.02 ± 0.03	1.97 ± 0.05			
Ground beetles	2008	0.35 ± 0.21	0.77 ± 0.19	3.69* (0.04)	9.91* (0.01)	0.27 (0.76)
	2009	0.69 ± 0.15	1.39 ± 0.10			
	2010	0.81 ± 0.31	1.26 ± 0.21			
Rove beetles	2008	1.61 ± 0.03	1.29 ± 0.22	7.61* (0.00)	0.27 (0.61)	0.24 (0.79)
	2009	1.17 ± 0.44	1.24 ± 0.24			
	2010	0.45 ± 0.27	0.33 ± 0.33			

Means were compared by two-way ANOVA, and the *F* calculated for the two factors (year and treatment) and their interaction. Significant values ($P < 0.05$) are marked with *

[a] Data were pooled across sampling dates within plots

Both spider's richness and diversity did not significantly differ between treatments and among years (Table 7). A total of fourteen species of ground beetles were collected in pitfall traps in the three-year field study. Five species accounted for 84% of the total number of individuals collected: *Pseudoophonus rufipes* DeGeer, *Distichus planus* Bonelli, *Paratachys elongatulus* Dejean, *Poecilus purpurascens* Dejean and *Microlestes corticalis* Dufour (Fig 3). There were significant differences in the activity-density of *D. planus* depending on the year ($F_{2, 48}$ = 12.04, P < 0.001), treatment ($F_{1, 48}$ = 15.44, P < 0.001) and `year by treatment' interaction ($F_{2, 48}$ = 9.62, P < 0.001). Likewise, significant changes were recorded for *P. rufipes* among years ($F_{2, 48}$ = 48.81, P < 0.001) and between treatments ($F_{1, 48}$ = 5.83, P < 0.001), and for *P. purpurascens* between treatments ($F_{1,48}$ = 6.37, P < 0.015). Richness values for ground beetles were significantly affected by treatment, but not by year, whereas diversity values significantly differ between treatments and among years (Table 7). In those cases where treatment was a significant factor, the estimated parameter was higher under the glyphosate herbicide regime.

Twelve different species of rove beetles were recorded in the pitfall traps along the three-year field survey. Among them, seven species account for 83% of the total: *Platysthetus nitens* Sahlberg, *Atheta lindbergiella* Brundin, *Aleochara olivieri* Fauvel, *Carpelimus rivularis* Motschulsky, *Carpelimus corticinus* Gravenhorst, *Amischa nigrofusca* Stephens and *Carpelimus parvulus* Mulsant et Rey (Fig 3). No significant differences in activity-density were detected between the two treatments in any of these species, whereas significant changes among years were found for *A. olivieri* ($F_{2, 48}$ = 4.07, P = 0.023), *P. nitens* ($F_{2, 48}$ = 4.40, P = 0.018) and *A. nigrofusca* ($F_{2, 48}$ = 3.45, P = 0.040). Richness and diversity values were significantly lower in 2010 than in the other two sampling years because of the limited catches of rove beetles in pitfall traps, but no significant differences were found between treatments (Table 7). Only three species of earwigs were collected during the three-year field survey: *Labidura riparia* (57%),

Nala sp. (40%) (Dermaptera: Labiduriidae) and *Labia sp*. (Dermaptera: Labiidae) (3%). Therefore, richness (S) and diversity indices were not calculated. No significant differences in activity-density were detected for any of these species between the two treatments, and only *L. riparia* showed significant changes among years ($F_{2, 48} = 121.15$, $P < 0.001$). No correlation was shown between the activity-density of the main predatory groups and the density of weeds, when both treatments (glyphosate and conventional) were considered in the Pearson's correlation analysis. However, a significantly positive correlation was recorded between total weed density and ground beetle richness and diversity ($r = 0.41$, $P = 0.04$ and $r = 0.54$, $P < 0.01$, respectively). When considering each treatment separately, weed density was positively correlated to spiders' activity-density, richness and diversity ($r = 0.73$, $P < 0.01$; $r = 0.73$, $P < 0.01$ and $r = 0.60$, $P = 0.04$, respectively) and to carabids' diversity ($r = 0.68$, $P = 0.02$) in plots treated with glyphosate.

Conclusion

This study suggest that weed control strategies with exclusive reliance on repeated glyphosate applications can reduce weed diversity and induce a shift in weed species composition in GT cotton. No significant differences were observed in the activity-density of ground-dwelling predators between conventional and glyphosate herbicide regimes, though an increase in ground beetles species richness and diversity was observed in glyphosate treated cotton. Yet, to prevent a reduction in weed diversity while maintaining some weed abundance it is crucial to avoid using the same herbicide year after year and to rotate herbicides and crops.

Assessment and conclusion

Assessment and conclusion by applicant:
Not relevant by title/abstract: The article is dealing with transgenic crop, which is not relevant for the glyphosate EU renewal.

Assessment and conclusion by RMS:

This study, performed in the EU in cotton, investigates the relationship between weed management and the beneficial predatory arthropods in a glyphosate-tolerant (GT) cotton crop. The objective of this long-term field-scale study is to provide insight into the impact of GT cotton crop subjected to two different weed management regimes on weed and ground-dwelling predatory communities. Conventional regime consisting on pre- and post-emergence herbicide applications (according to the recommended regional practices for cotton production) and another one based on glyphosate only application in post emergence were compared. Changes in the density, species richness, diversity and seasonal phenology of plants and predator communities under both herbicide regimes have been assessed in a three-year farm-scale study in southern Spain.

During the first 2 years (2008 and 2009), no significant differences were observed in weed diversity between herbicide management regimes. In the last year (2010) diversity was higher under the conventional herbicide management. According to the study authors this was due to the different herbicide use:

- a residual herbicide in pre-emergence that allows the presence of *E. colona* until the application of a post-emergence graminicide;
- and a control with post-emergence treatments with glyphosate, leading to a shift in weed species composition, where *P. oleracea* became the almost only weed present in the field.

Significant differences between treatments were obtained for ground beetles species richness and diversity and a positive correlation with weed density could be established for both parameters. This study suggests that higher weed biomass can sustain a greater number of prey species. Since total weed density was higher under glyphosate herbicide regime, ground beetles can have a wider target spectrum in these plots than in the conventional treated ones. In addition, the shift in favour to *P. oleracea* in plots managed with glyphosate, could also be related with the positive correlation between weed density and the diversity of carabids in these plots.
Significant reduction in ground beetles, rove beetles and earwigs and a significant increase of spiders through the three years of the study in both conventional and glyphosate herbicide regimes were observed. Similarly, significant differences for rove beetles richness and diversity and ground beetles diversity were also obtained among years, which in the case of rove beetles also showed a reduction through the three years of the study. This study suggests that interspecific competition may occur between these groups.

Overall, glyphosate (applied post-emergence) in this three-year farm-scale study resulted in a shift in weed species composition, suggests a positive correlation between weed density and the diversity of carabids and interspecific competition may occur between predatory groups

This study is considered relevant for biodiversity and indirect effect issues. However its relevance is limited as it focusses on post-emergence glyphosate applications and results were compared to an other herbicide treatment only. So the differences observed in the study are very likely the consequence of the different timing of application.
Based on the above, the study is considered of limited relevance.

RMS notes that insecticides were applied (at sowing). Herbicides treatments (other than glyphosate) were also applied in conventional managed plots. Their direct (toxic) effect was not investigated.
Drought during 2009 (rainfall between May and August was 8.8 mm in comparison with the average of 58 mm in the last 30 years) may also have reduced herbicide effectiveness (by reducing absorption, translocation and metabolism of herbicides).
The study is relevant for for biodiversity and indirect effect issues and considered reliable with restrictions.

B.9.7.2. Published literature studies identified by RMS as less relevant but supplementary and reliable or reliable with restrictions after detailed assessment of full-text

Data point:	CA 9
Report author	Mudge J. F. *et al.*
Report year	2019
Report title	Wetland macrophyte community response to and recovery from direct application of glyphosate-based herbicides
Document No	Ecotoxicology and Environmental Safety, (2019) Vol. 183, Art. No. 109475
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by title/abstract

Full summary of the study according to OECD format

Community-scale impacts of glyphosate-based herbicides on wetland plant communities and the magnitude of those impacts that should be considered biologically relevant are poorly understood. The researchers contrast three different thresholds for setting biologically meaningful critical effect sizes for complex ANOVA study designs. Each of the critical effect sizes was used to determine optimal α levels for assessment of how different concentrations of glyphosate-based herbicides affect wetland plant communities over two years of herbicide application (alone and in combination with agricultural fertilizers) and two subsequent years without herbicide (or fertilizer) application. The application of glyphosate-based herbicides was found to result in a decrease in macrophyte species richness, an increase in macrophyte species evenness, a decrease in macrophyte cover and a reduction in community similarity. There was little evidence that nutrient additions directly or indirectly affected plant community endpoints. The glyphosate effects were evident in the first year of herbicide application in 2009, and became more pronounced in the second year of herbicide application in 2010. However, when herbicides were not applied in 2011, recovery was observed in most endpoints, with the exception being species evenness, for which partial recovery was not observed until 2012. Optimal α levels differed among the three critical effect sizes for each ANOVA term and endpoint combination, however regardless of differences in α levels, conclusions were generally consistent across all critical effect sizes.

Materials and methods

Study location

The field site was located in the Long Term Experimental Wetlands Area (66°29′59.02″W, 45°40′48.62″N) about 60 km from the city of Saint John in southern New Brunswick, Canada. It is a mixed-wood Acadian forest, dominated by red spruce (Picea rubens), white spruce (Picea glauca), red maple (Acer rubrum), white birch (Betula papyrifera) and yellow birch (Betula alleghaniensis). The six km^2 study area was mechanically cleared of trees and the top soil pushed into berms in 1997 and 1998. Wetlands formed naturally in depressions and next to the windrows because of the disruption of drainage patterns of the area. This created a landscape containing hundreds of small wetlands ranging from<1 ha to several hectares in size, which have become naturalized to varying extent since their creation. Within this area 24 wetlands intended to be representative of wetlands located in and around agricultural fields

were chosen, that were relatively small (< 1 ha), had no permanent inflow or outflow, and had relatively homogenous macrophyte cover.

Experimental design

The study was designed as a replicated split-plot experiment. Each experimental wetland was bisected by an impermeable plastic barrier and one half of the system was randomly assigned as a control while the other half was assigned the treatment. Bisections were oriented along the shortest possible diameter through the wetland with similar topography, habitat features and plant community composition on each side. In two wetlands with suspected transient flow patterns (usually only in spring), the inflow side was designated control and the outflow side was treated. This prevented contamination of the control side of the wetland if the integrity of the barrier was compromised during the spring because the control side water flowed into the treatment side rather than the reverse. This split-plot design, where each wetland is partitioned into a control and an experimental area, minimizes the problem of confounding the natural variability among ponds with treatment effects.
During the late summer of 2008 each of the 24 wetlands was split in half using an impermeable, stable, inert, UV resistant plastic barrier constructed from 0.76mm opaque black high density polyethylene (HDPE) (Poly-Flex Inc. Geomembrane Lining Systems, Grand Prairie, Texas). The bottom of the barrier was weighted with gravel and buried 10 cm into the bottom substrate and extended approximately 2m beyond the high water mark of each wetland. The barriers were approximately 1m in height to ensure that they extended above the high water level for every wetland. The integrity of the barrier was inspected in the spring and fall and periodically throughout all field seasons. Barrier maintenance was required in all wetlands at some point during the study, but there was no evidence of contamination with glyphosate levels consistently below detection limits in water samples from control halves (Edge et al., 2012).
The glyphosate-based herbicide Roundup WeatherMax™ (540 g acid equivalent (a.e.)/L, Monsanto, Winnipeg, MB, CAN) was applied at two different concentrations, alone and in combination with nutrients. This resulted in four treatment categories:

1. A low target aqueous glyphosate concentration (210 µg a.e./L) (L).
2. A high target aqueous glyphosate concentration (2, 880 µg a.e./L) (H). This is the direct overspray concentration expected in a 15 cm deep wetland with no intercepting vegetation that has been oversprayed at the maximum label rate (7.9 L/ha).
3. The low target aqueous glyphosate concentration together with nutrient additions (LN).
4. The high target aqueous glyphosate concentration together with nutrient additions (HN).

The two target concentrations represent the maximum environmentally observed concentrations in agricultural wetlands (L) and a reasonable worst case scenario assuming complete overspray of the entire wetland at the maximum label rate (H). The two treatments that combine herbicide and nutrients were done to simulate an agricultural multiple stressor scenario. The nutrient treatments were designed to increase the trophic status of each wetland one level (e.g. oligotrophic to mesotrophic) above background levels (calculated in 2008 – one year prior to chemical treatment). Each treatment (i.e. L, H, LN, HN) was replicated in the treatment half of 6 wetlands.

The herbicide was applied to the wetlands twice in the spring of 2009 and twice in the spring of 2010. In 2009 the first herbicide application occurred on 15/16 May and the second application on 9/10 June. In 2010 the first application occurred on 26/27 April and the second application occurred on 24/25 May. Two applications were made to simulate standard agricultural practices for glyphosate tolerant crops and timed to correspond with spring macrophyte emergence in each year. Post-application sampling of ponds showed that glyphosate concentrations were statistically significantly higher in the high concentration halves than the low concentration halves although it was not possible to precisely hit target concentrations. There was no evidence of contamination on the control sides of ponds (Edge et al., 2012). The 12 nutrient treatment wetlands were sprayed on the treatment half with nutrients commonly used in fertilizers (technical grade ammonium nitrate and phosphoric acid, purchased from Fisher Scientific).

In 2008, wetlands were generally classified as oligotrophic/mesotrophic based on aqueous nutrient concentrations (Wetzel, 2001), and nutrients were added to increase the aqueous total Kjeldahl nitrogen (TKN) concentrations by one trophic level, and phosphorus was added to maintain the TKN:TP ratios observed in 2008. Nutrients were added to wetlands four times in each of 2009 and 2010. Timing of nutrient additions was determined by monitoring weekly wetland water chemistry data and adding nutrients to maintain TKN and TP at their desired levels. In 2009 nutrients were added to wetlands on 14 May, 29 May, 3 July and 19 Aug. In 2010 nutrients were added to wetlands on 22/23 April, 13/14 May, 24/25 June, and 30/31 July.
Immediately before each application, total wetland volume was calculated from depth measurements taken at regularly spaced intervals along transects of each wetland. We then used the estimated wetland water volume to estimate the volume of herbicide/nutrient required to achieve a particular target concentration. The herbicide/nutrients were then mixed in ~3 L of wetland water and applied evenly to the surface of the wetland with a backpack sprayer. The same volume of water was applied to the control side using a separate backpack sprayer to ensure equivalent physical disturbance on both sides of each wetland.
The researchers estimated species composition of both emergent and submerged macrophyte species of each wetland half (Crow and Hellquist, 2000a, 2000b) and percent cover to the nearest 1% (Anderson, 1986) for each species maintaining the same search effort per unit area for each wetland half (approximately 3 s/m^2 on average). Plant surveys were conducted in late May, between the first and second herbicide treatments, and twice more after the second herbicide treatment, once in late June and once in August in 2009 and 2010. To assess recovery from herbicide and nutrient applications the researchers conducted plant surveys in late June, 2011, August 2011 and early August of 2012.

Data analysis

This study focuses on four plant community endpoints;

i) species richness,
ii) Pielou's species evenness index (Magurran, 2004),
iii) the sum of the peak cover for each species over the summer and
iv) the Bray-Curtis similarity index (Magurran, 2004) between the treated and control side of each wetland.

The researchers calculated community endpoints for each wetland half/year combination using two different techniques, maximum and mean cover, for pooling across the three samples. The sample containing the maximum cover of each species was used to estimate species richness and total cover for each wetland half in each year. The average cover of each species across all three samples was used to estimate evenness and community similarity. Thus, species richness for a wetland half was the count of all species that were detected in at least one of the surveys in any year. Total cover was calculated by summing the maxima for each species. When the largest observed abundance of a species across all three surveys was<1%, the maximum abundance for that species was assumed to be 0.25% (the midpoint between 0% and 0.5%, which would round up to 1%). The Pielou's species evenness was calculated as

$$\text{Evenness} = \sum_{i=1}^{S} p_i * \ln(p_i) \Big/ \ln(S),$$

where S is the total number of species in the community and p is the proportion of the total cover that is comprised of the cover of species i.
The Bray-Curtis similarity between wetland halves was calculated as

$$2 * \sum_{i=1}^{S} minCoverTC_i \Big/ (TotalCover_T + TotalCover_C),$$

where S is all species in common between the treatment and control halves of a pond, *minCoverTCi* is the cover of species i on the side where it was lowest of the treatment and control sides, *TotalCoverT* is the total macrophyte cover on the treatment side and *TotalCoverC* is the total macrophyte cover on the control side.
Species evenness and community similarity range between 0 and 1 so were arcsine transformed prior to statistical analysis. The researchers used partially nested repeated measures linear mixed effect Type III

ANOVA models to examine the influence of wetland half (treated vs. control), in combinations with herbicide concentration, nutrient concentration, and/or year, on species richness, Pielou's species evenness, and max. total cover. Herbicide concentration and nutrient treatments were each considered between subjects fixed factors, with pond as an incomplete random blocking factor, side (treated or control) as a within-subjects fixed factor and year as the within subjects repeated measure random factor. For the Bray-Curtis similarity endpoint, side was not possible to include as a factor because it was incorporated into the similarity calculation.
Thus, the researchers used an analogous ANOVA model to examine the influences of different combinations of herbicide concentration (between subjects fixed factor), nutrient treatment (between subjects fixed factor) and time (within subjects random repeated measure factor) on Bray-Curtis similarity.
Optimal α: Statistical significance of ANOVA terms was evaluated using the optimal α approach for minimizing the combined probabilities or costs of Type I and II errors in null Hypothesis tests (Mudge et al., 2012). The calculation of optimal α levels for ANOVA terms requires estimates of the relative costs of Type I and II errors, the relative prior probabilities of null and alternate hypotheses, the numerator and denominator degrees of freedom for the ANOVA term, and an estimate of the critical (i.e. biologically relevant) effect size. As there was no strong rationale for considering one type of error to be more serious than the other or to assume different prior probabilities of the null and alternate hypotheses, the researchers set the relative costs of Type I and II errors and prior probabilities to be equal.

Critical effect size

Critical effect sizes can be difficult to conceptualize in ANOVA designs because researchers are often interested in pairwise differences among groups while effects in ANOVA designs usually incorporate variability across all groups, rather than pairwise differences. In this study, the researchers used (1) the η^2 approach, (2) the max. – min./within-group σ approach and (3) the max. % difference from control mean approach. All 3 effects sizes must be converted to Cohen's f^2 to be implemented (Cohen, 1988). The η^2 critical effect size measure (Cohen, 1988) is analogous to R^2 in that it represents the proportion of the total variance in the ANOVA model that is explained by group membership (either the factor levels or all the combinations of multiple factor levels for interaction terms). The reserchers chose $\eta^2=0.2$ as the minimum biologically relevant proportion of total variance explained by group membership. $f^2=\eta^2/1-\eta^2$ therefore $f^2=0.25$ for each endpoint and each ANOVA term.
The maximum group mean – minimum group mean/within-group σ critical effect size is analogous to the Cohen's d critical effect size used as an effect size measure in t-tests (the difference between group means relative to the within-group standard deviation) in that it represents the smallest possible among-group variance that can produce a maximum difference between any two group means at least as large as some specified multiple of the within-group standard deviation (f^2 = max. μ – min. μ/within-group σ*2 * k, where k is the number of groups). The minimum biologically relevant difference between any two groups was chosen to be where max. μ – min. μ/within-group σ equals 2. This critical effect size value was chosen to be consistent with an effect size of 2 standard deviations proposed by Munkittrick et al. (2009) for a wide variety of biological and ecological endpoints. Because this critical effect size incorporates the number of groups the critical effect size will be smaller for factors with more groups, all other things being equal.
The max. % difference from the control mean critical effect size represents the smallest possible among-group variance that can produce a maximum difference between any two group means at least as large as some specified percent difference from the control mean. The first two effect sizes were variance–standardized but max. % difference is not, and thus requires an estimate of both the expected control mean and the expected within-group variance. The estimates of the control mean and the within-group variance were taken from the observed data. For each ANOVA term, the max. % difference from the control mean critical effect size was converted to an f^2 critical effect size by: (1) multiplying the control mean by 1 ± the critical maximum % difference from the control mean/100 to get the minimum and maximum of group means, (e.g. control mean = 5; critical maximum % difference = 25; 5*1 ± 25/100 = 3.75 and 6.25) (2) calculating the minimum among group variance for the k group means by assuming all other group means are clustered at the midpoint between the minimum and maximum, then (3) dividing the minimum among-group variance (from Step 2) by the within-group variance from the data.

The minimum biologically relevant difference between any two groups was chosen to be a 25% difference from the control mean. This critical effect size value was also recommended by Munkittrick et al. (2009) for a variety of environmental monitoring endpoints.

Optimal α and associated β levels were calculated for each of the three potential critical effect sizes using an iterative process described in Mudge et al. (2012) (Table 1).

The different critical effect sizes and degrees of freedom for each endpoint and ANOVA term combination produce different optimal α levels. Tests associated with larger optimal α levels imply weaker experimental design and thus, weaker evidence for effects (or lack thereof) than results associated with smaller optimal α levels. The researchers classified the different optimal α levels into three categories corresponding to different levels of confidence in the statistical outcome. For optimal $\alpha \leq 0.05$, they considered any p-values$\leq\alpha$ to constitute strong evidence for an effect and any p-values $> \alpha$ to be strong evidence that the effect was smaller than the critical effect size. For optimal α between 0.05 and 0.15, they considered any p-values$\leq\alpha$ to be moderate evidence for an effect and p-values $> \alpha$, to be moderate evidence that the effect was smaller than the critical effect size. Finally, for optimal alpha>0.15 they considered any p-value$\leq\alpha$ to be weak evidence for an effect, and a p-value $> \alpha$ to be weak evidence that the effect was smaller than the critical effect size.

Critical effect sizes and optimal α levels

After translating each of the three critical effect size measure into f^2 values, each ANOVA term and endpoint combination had at least two different potential critical effect size values – occasionally two different critical effect size measures resulted in the same critical effect size value (22 of 31 ANOVA term and endpoint combinations had three different critical effect size values) (Table 1). There were eight ANOVA term and endpoint combinations for which the $\eta^2 \geq 0.2$ and the max. diff. $\geq 2\sigma_{(\text{within-group})}$ critical effect sizes corresponded to the same f^2 value and one case where the $\eta^2 \geq 0.2$ and max. diff.$\geq 25\%\mu_{(\text{control})}$ critical effect sizes corresponded to nearly the same f^2 value. The $\eta^2 \geq 0.2$ critical effect size (corresponding to at least 20% of the total variance in the data being explained by the factor level membership) translated to an among-group/within-group variance ratio of $f^2=0.25$ (Table 1, Fig. 1).

Table 1

Critical effect sizes expressed as a ratio of among-group variance to within-group variance (f2), optimal α and associated β levels for each endpoint and ANOVA term combination under three different approaches to selecting a critical effect size. (values in bold, regular and italic font are strong, moderate and weak evidence respectively).

endpoint	ANOVA term	f^2	$\eta^2 \geq 0.2$		max diff. $\geq 2\sigma_{(\text{within group})}$			max diff. $\geq \pm 25\%\mu_{(\text{control})}$		
			α	β	f^2	α	β	f^2	α	β
	side	0.25	*0.18*	*0.22*	1.0	**0.033**	**0.028**	0.26	*0.18*	*0.21*
	side*concentration	0.25	*0.22*	*0.25*	0.50	0.13	0.13	0.13	*0.29*	*0.37*
	side*nutrients	0.25	*0.22*	*0.25*	0.50	0.13	0.13	0.13	*0.29*	*0.37*
richness	side*concentration*nutrientsside*year	0.25	0.220.018	*0.25*	0.25	*0.22*	*0.25*	0.064	*0.34*	*0.47*
		0.25		**0.019**	0.25	**0.018**	**0.019**	0.064	*0.19*	*0.23*
	side*year*concentration	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.032	*0.29*	*0.36*
	side*year*nutrients	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.032	*0.29*	*0.36*
	side*year*concentration*nutrients	0.25	**0.018**	**0.019**	0.063	*0.20*	*0.24*	0.016	*0.35*	*0.45*
	Side	0.25	*0.18*	*0.22*	1.0	**0.033**	**0.028**	2.7	**0.0021**	**0.0013**
	side*concentration	0.25	*0.22*	*0.25*	0.50	0.13	0.13	1.3	**0.026**	**0.020**
	side*nutrients	0.25	*0.22*	*0.25*	0.50	0.13	0.13	1.3	**0.026**	**0.020**
evenness	side*concentration*nutrientsside*year	0.25	0.220.018	*0.25*	0.25	*0.22*	*0.25*	0.67	0.088	0.083
		0.25		**0.019**	0.25	**0.018**	**0.019**	0.67	**0.00010**	**0.000085**
	side*year*concentration	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.34	**0.0058**	**0.0061**
	side*year*nutrients	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.34	**0.0058**	**0.0061**
	side*year*concentration*nutrients	0.25	**0.018**	**0.019**	0.063	*0.20*	*0.24*	0.17	**0.050**	0.057
	side	0.25	*0.18*	*0.22*	1.0	**0.033**	**0.028**	0.31	0.15	*0.18*
	side*concentration	0.25	*0.22*	*0.25*	0.50	0.13	0.13	0.16	*0.27*	*0.34*
	side*nutrients	0.25	*0.22*	*0.25*	0.50	0.13	0.13	0.16	*0.27*	*0.34*
cover	side*concentration*nutrientsside*year	0.25	0.220.018	*0.25*	0.25	*0.22*	*0.25*	0.078	*0.33*	*0.45*
		0.25		**0.019**	0.25	**0.018**	**0.019**	0.078	*0.16*	*0.19*
	side*year*concentration	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.039	*0.26*	*0.33*
	side*year*nutrients	0.25	**0.018**	**0.019**	0.13	0.088	0.10	0.039	*0.26*	*0.33*
	side*year*concentration*nutrients	0.25	**0.018**	**0.019**	0.063	*0.20*	*0.24*	0.019	*0.34*	*0.43*
	concentration	0.25	*0.16*	*0.19*	1.0	**0.022**	**0.019**	0.64	0.054	0.051
	nutrients	0.25	*0.16*	*0.19*	1.0	**0.022**	**0.019**	0.64	0.051	0.051
	concentration*nutrients	0.25	*0.16*	*0.19*	0.50	0.077	0.078	0.32	0.13	0.15
similarity	year	0.25	0.063	0.068	0.50	**0.011**	**0.011**	0.32	**0.039**	**0.041**
	concentration*year	0.25	0.063	0.068	0.25	0.063	0.068	0.16	0.12	0.14
	nutrients*year	0.25	0.063	0.068	0.25	0.063	0.068	0.16	0.12	0.14
	concentration*nutrients*year	0.25	0.063	0.068	0.13	*0.16*	*0.19*	0.079	*0.22*	*0.28*

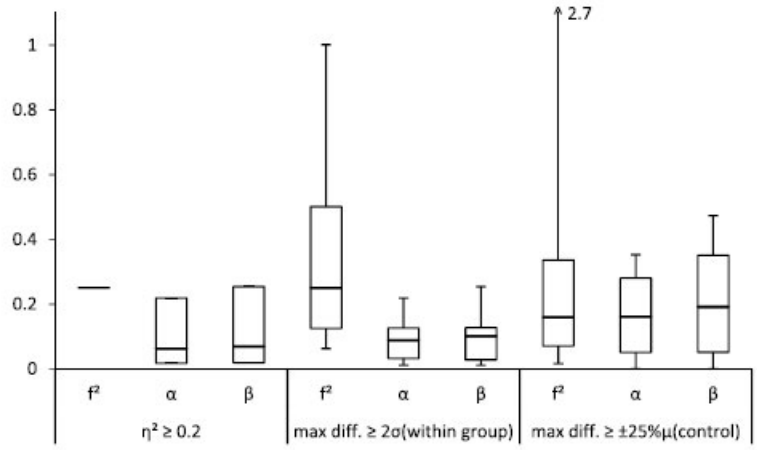


Fig. 1. Critical effect sizes (standardized as f^2 values, the ratio of the size of the among-group variance to the within-group variance), optimal α and associated β levels summarized over each ANOVA term and endpoint (richness, evenness, cover and similarity) combination, for each of the three critical effect size measures.

The median f^2 converted critical effect size value for the max. diff. $\geq 2\ \sigma_{(within\text{-}group)}$ measure was also 0.25, with larger converted f^2 values for the ANOVA terms containing few groups (side, side*concentration and side*nutrients) and smaller converted f^2 values for ANOVA terms containing many groups (side * year * concentration, side * year * nutrients, and side * year * concentration * nutrients). This is because the among group variance required to produce a constant maximum difference between any 2 groups decreases as the number of groups increases (additional group means added to the midpoint between the minimum and maximum would decrease the variance, holding the difference between the minimum and maximum groups constant). The max. diff. $\geq 25\%\mu_{(control)}$ critical effect size measure translated to f^2 values with a lower median value of 0.16. This effect size measure also had the largest range of f^2 critical effect sizes (0.02–2.7) because the f^2 critical effect size was dependent not only on the number of groups contained within each ANOVA term, but also on the size of a 25% difference from the control mean relative to the within-group variance. Optimal α levels ranged from 0.00010 to 0.35 among the ANOVA terms for the three potential critical effect sizes, with a mean α level of 0.13, a mean β level of 0.15 and a mean average of α + β of 0.14 (Table 1). Where the researchers use $\alpha > 0.05$ they increase the risk of committing a Type I error over the standard practice of using $\alpha=0.05$, but this is more than offset by the reduced risk of committing a Type II error.

Results

Species richness

A total of 98 different taxa were found during plant surveys. The effect of wetland side on species richness differed among years for each of the three potential critical effect sizes (Table 2, Fig. 2). Richness was lower on treated sides than on control sides in 2009 and 2010, but in the 2011 and 2012 years when the treated sides did not receive further treatment, the sides had similar richness. For the $\eta^2 \geq 0.2$ and max. diff $\geq 2\sigma_{(within\ group)}$ critical effect sizes, the α levels for the side*year interaction were each 0.018, and the observation of p-values smaller than these low α levels indicated strong evidence for a real effect.

By contrast, there was only weak evidence of a side*year interaction for the max. diff $\geq 25\%\mu$(control) critical effect size, with $p < \alpha=0.19$. The consistently significant interaction between side and year for all potential critical effect sizes precluded interpretation of the effect of side independent of the effect of year.

Table 2
Degrees of freedom, observed effect sizes and p-values of the ANOVA for the difference in species richness between wetland sides. P-values not in bold were not significant using optimal α with any of the three potential critical effect sizes.

	df	F-value	p-value
intercept	1, 120	691.001	< 0.0001
side	1, 17	4.912	**0.0406*‡**
side*concentration	2, 17	1.019	0.382
side*nutrients	2, 17	0.053	0.9485
side*concentration*nutrients	2, 17	0.317	0.7325
side*year	6, 120	55.069	**< 0.0001*†‡**
side*year*concentration	6, 120	0.909	0.4912
side*year*nutrients	6, 120	0.887	0.507
side*year*concentration*nutrients	6, 120	0.584	0.7425

*p-value ≤ optimal α for the η2 ≥ 0.2 effect size.
†p-value ≤ optimal α for the max diff. ≥ 2σ(within group) effect size.
‡p-value ≤ optimal α for the max diff. ≥ ± 25%μ(control) effect size.

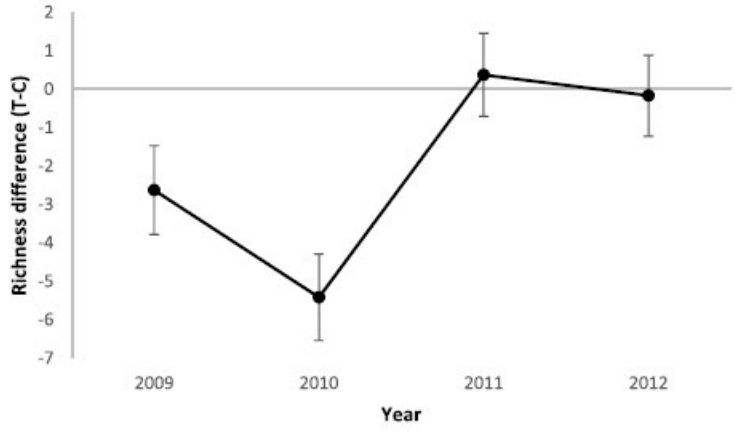


Fig. 2. Mean differences in species richness (± 1 SE) between treated and control sides of wetlands from 2009 to 2012.

Species evenness

The effect of wetland side on species evenness also differed among years, regardless of which of the three potential critical effect sizes was used (Table 3, Fig. 3).
The mean difference in species evenness between treated and control sides of wetlands increased from 0.038 to 0.049 from 2009 to 2011 (the year following the two years in which the treatments were applied), but in 2012, the mean difference dropped to 0.014. This observed effect constitutes strong evidence of a real side*year effect for species evenness using each of the three potential critical effect sizes, as the p-value was less than the already low optimal α levels of 0.018, 0.018 and 0.00010 for the $\eta^2 \geq 0.2$, max. diff $\geq 2\sigma_{(\text{within group})}$ and max. diff $\geq 25\%\mu_{(\text{control})}$ critical effect sizes, respectively. The significant interaction between side and year for all potential critical effect sizes precluded interpretation of the effect of side independent of the effect of year.

Table 3
Degrees of freedom, observed effect sizes and p-values of the ANOVA for the difference in Pielou's species evenness between wetland sides. P-values not in bold were not significant using optimal α with any of the three potential critical effect sizes.

	df	F-value	p-value
intercept	1, 120	3416.889	< 0.0001
side	1, 17	18.05	**0.0005*†‡**
side*concentration	2, 17	0.535	0.5952
side*nutrients	2, 17	1.046	0.3728
side*concentration*nutrients	2, 17	0.464	0.6366
side*year	6, 120	5.862	**< 0.0001*†‡**
side*year*concentration	6, 120	0.349	0.9093
side*year*nutrients	6, 120	0.648	0.692
side*year*concentration*nutrients	6, 120	1.096	0.369

*p-value ≤ optimal α for the $\eta 2 \geq 0.2$ effect size.
†p-value ≤ optimal α for the max diff. ≥ 2σ(within group) effect size.
‡p-value ≤ optimal α for the max diff. ≥ ± 25%μ(control) effect size.

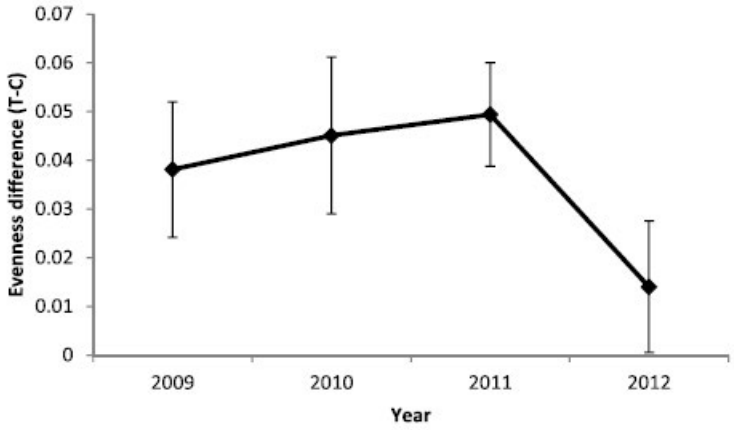


Fig. 3. Mean differences in Pielou's species evenness (± 1 SE) between treated and control sides of wetlands from 2009 to 2012.

Macrophyte cover
The factors influencing macrophyte cover depend on what critical effect size is used. For the max. diff ≥ $2\sigma_{(within\ group)}$ and max. diff ≥25%$\mu_{(control)}$ critical effect sizes, there was weak evidence of a side*year*concentration*nutrients effect (Table 4). The significant side * year * concentration * nutrient interaction represented the larger mean differences between treated and control sides of H wetlands relative to other wetland treatments (HN, L, and LN) in 2010 (Fig. 4). For the larger, $\eta^2 \geq 0.2$ critical effect size, there was strong evidence suggesting that the side*year*concentration*nutrient interaction effect does not explain at least 20% of the variability in macrophyte cover, $p>\alpha$ and $\beta = 0.019$.
There was, however, strong evidence for a side*year interaction effect under the $\eta^2 \geq 0.2$ critical effect size ($p < \alpha = 0.018$) whereby the macrophyte cover was lower on treated sides than control sides during years receiving treatments (2009 and 2010) but not in the following years (2011 and 2012), and weak evidence for a side*- concentration*nutrient interaction effect under the $\eta^2 \geq 0.2$ critical effect size ($p < \alpha = 0.22$), with H wetlands having slightly less macrophyte cover on treated sides relative to their controls compared to all other treatment categories (HN, L, and LN). The significant side*year*concentration*nutrient interaction for the max. diff ≥ $2\sigma_{(within\ group)}$ and max. diff ≥25%$\mu_{(control)}$ critical effect sizes precluded interpretations of main effects, 2-way and 3-way interactions, while the significant side*year and side*concentration*nutrient interactions for the $\eta^2 \geq 0.2$ critical effect size precluded interpretation of the main effects.

Table 4
Degrees of freedom, observed effect sizes and p-values of the ANOVA for the difference in the sum of maximum macrophyte cover for each species between wetland sides. P-values not in bold were not significant using optimal α with any of the three potential critical effect sizes.

	df	F-value	p-value
intercept	1, 120	1132.551	< 0.0001
side	1, 17	46.3185	**< 0.0001*†‡**
side*concentration	2, 17	0.7718	0.4777
side*nutrients	2, 17	0.3446	0.7133
side*concentration*nutrients	2, 17	2.0251	**0.1626*†‡**
side*year	6, 120	14.2379	**< 0.0001*†‡**
side*year*concentration	6, 120	0.6355	0.7016
side*year*nutrients	6, 120	1.8215	**0.1004‡**
side*year*concentration*nutrients	6, 120	2.2881	**0.0398†‡**

*p-value ≤ optimal α for the $\eta 2 \geq 0.2$ effect size.
†p-value ≤ optimal α for the max diff. ≥ 2σ(within group) effect size.
‡p-value ≤ optimal α for the max diff. ≥ ± 25%μ(control) effect size.

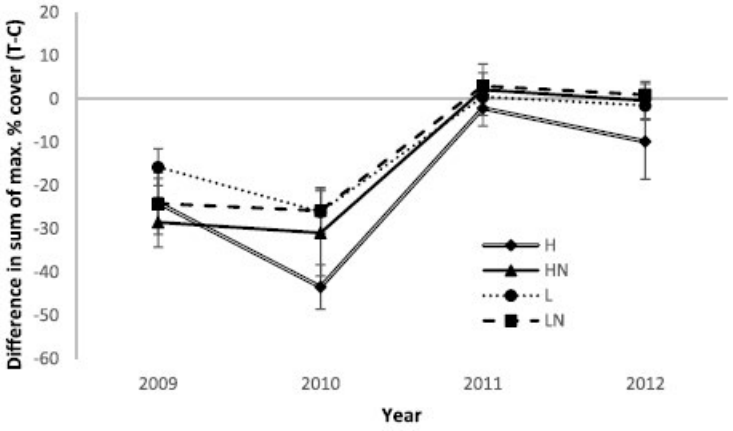


Fig. 4. Mean differences in the sum of the maximum percent cover for each species (± 1 SE) between treated and control sides of wetlands from 2009 to 2012. The double line with diamond markers represents wetlands treated with the high concentration of the glyphosate herbicide on one side, the solid line with triangle markers represents wetlands treated with the high concentration of the glyphosate herbicide plus nutrients on one side, the dotted line with diamond markers represents wetlands treated with the low concentration of the glyphosate herbicide and the dashed line with square markers represents wetlands treated with the low concentration of the glyphosate herbicide plus nutrients on one side.

Similarity in community composition

Averaged across years, there was weak evidence for a difference in Bray-Curtis similarity between the H and L treatment concentrations under the $\eta^2 \geq 0.2$ critical effect size ($p < \alpha = 0.16$) (Table 5). Wetlands receiving the high glyphosate treatment had a mean similarity between control and treated sides of 62.0%, while wetlands receiving the low glyphosate concentration had a mean similarity between control and treated sides of 69.0%. There was also moderate evidence (critical effect size $\eta^2 \geq 0.2$, $p < \alpha = 0.063$) to strong evidence (critical effect sizes max. diff$\geq 2\sigma_{(\text{within group})}$ and max. diff$\geq 25\%\mu_{(\text{control})}$, $p < \alpha = 0.011$ and $p < \alpha = 0.039$ respectively) for a significant difference in Bray-Curtis similarity among years. This difference in similarity among years is primarily due to a lower observed Bray-Curtis similarity between wetland sides in 2010, relative to 2009, 2011 and 2012 (Table 5, Fig. 5).

Table 5
Degrees of freedom, observed effect sizes and p-values of the ANOVA for Bray-Curtis similarity in community composition between wetland sides. P-values not in bold were not significant using optimal α with any of the three potential critical effect sizes.

	df	F-value	p-value
intercept	1, 60	2658.3748	< 0.0001
concentration	1, 20	4.0712	**0.0572***
nutrients	1, 20	0.2241	0.6411
concentration*nutrients	1, 20	0.5823	0.4543
Year	3, 60	6.501	**0.0005*†‡**
concentration*year	3, 60	0.1958	0.8988
nutrients*year	3, 60	1.2276	0.3076
concentration*nutrients*year	3, 60	0.2778	0.8412

*p-value ≤ optimal α for the η2 ≥ 0.2 effect size.
†p-value ≤ optimal α for the max diff. ≥ 2σ(within group) effect size.
‡p-value ≤ optimal α for the max diff. ≥ ± 25%μ(control) effect size.

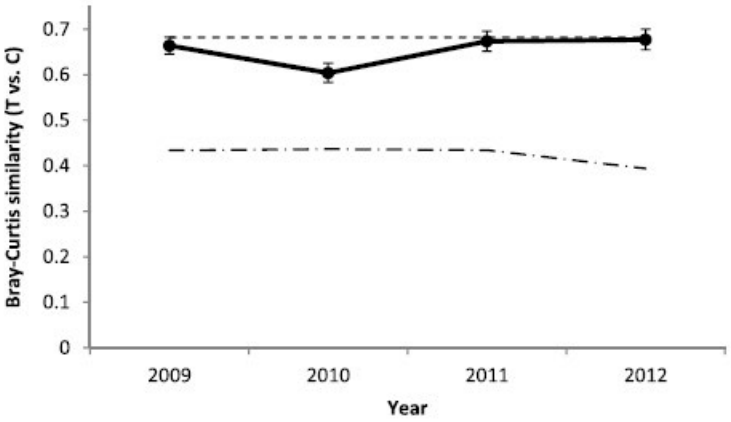


Fig. 5. Mean Bray-Curtis similarity (± 1 SE) between treated and control sides of wetlands in 2009–2012 (solid line). For comparison, the upper dashed line represents the mean control side similarity between two consecutive years, and the lower dashed line represents the mean control side similarity between wetlands for each year.

Conclusion

(1) Glyphosate application reduced species richness and % cover by at least 25% relative to the control side.
(2) Glyphosate application increased evenness by, at least, 25% relative to the control sides.
(3) All impacts of glyphosate applications in 2009 and 2010 were dramatically reduced or absent by 2012.
(4) There was no evidence of concentration-dependent effects or interactive effects of nutrient applications.
(5) In most tests, the targeted effect size did not affect statistical significance but occasionally evidence for small to medium effects was found but there was not evidence for large effects.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by title/abstract: Long term monitoring study specific to Canada Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). The formulation tested is not the representative formulation for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.

Assessment and conclusion by RMS:

The objective was the assessment of how different concentrations of glyphosate-based herbicides affect wetland plant communities over two years of herbicide application (alone and in combination with agricultural fertilizers) and two subsequent years without herbicide (or fertilizer) application. Lingering effects in the years after herbicides were applied (i.e. recovery) were also investigated.

The application of glyphosate-based herbicides was found to result in a decrease in macrophyte species richness, an increase in macrophyte species evenness, a decrease in macrophyte cover and a reduction in community similarity. These effects were evident in the first year of herbicide application in 2009, and became more pronounced in the second year of herbicide application in 2010. However, when herbicides were not applied in 2011, recovery was observed in most endpoints, with the exception being species evenness, for which partial recovery was not observed until 2012.

RMS notes that the study did not examine changes at the individual species level. The study is then not relevant for use in the risk assessment but nevertheless may address biodiversity/indirect effect issues.

RMS however notes that significant reduction in plant cover on treated sides of wetlands relative to their control sides following glyphosate herbicide application is not surprising, as reducing plant cover is the intended purpose of glyphosate herbicides. The lack of a concentration dependent effect in this study likely results from the additional spray at the maximum recommended label rate targeted specifically at emergent macrophytes that all wetlands received immediately following the application of the target concentration to the water. The purpose of the additional glyphosate application directly targeting the macrophyte community was to maximize the possibility of indirect impacts of glyphosate herbicides on the invertebrate or amphibian communities through direct effects to the plant community. However, this consistent amount of herbicide applied directly to the plant community on the treated sides of all wetlands was also much higher than the dose received through the different treatment concentrations applied directly to the water's surface.
This study would be then relevant only for aquatic uses when emergent macrophytes are directly exposed.

The study is of limited relevance as the exposure of emergent macrophytes (directly sprayed) was considerably higher than expected from a contamination via run-off/drift.

Due to low relevance RMS did not assess the reliability in depth. Nevertheless, RMS notes that this experimental study was also used and described in Baker et al, 2014, 2016 (also assessed by RMS). In both of them, it was highlighted that measured concentrations of glyphosate were far below those targeted and fastly decreased in the wetlands.

B.9.8. EFFECTS ON BIOLOGICAL METHODS FOR SEWAGE TREATMENT

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B.9.9. MONITORING DATA

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B.9.10. BIOLOGICAL ACTIVITY OF METABOLITES POTENTIALLY OCCURRING IN GROUNDWATER

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B.9.11. STUDIES EXCLUDED BY RMS AFTER DETAILED ASSESSMENT OF FULL-TEXT

The studies presented thereafter are the ones for which the applicant has submitted a study summary and assessment. They are also listed in the Volume 3 CA B.9 in Table B.9.11.1.4-2 (together with the studies for which only full-text but no summary and detailed analysis was available (data gap has been identified for several of them)).

1. Birds

Data point	CA 9
Report author	Roongruangchai J. *et al.*
Report year	2018
Report title	The teratogenic effects of glyphosate based herbicide (GBH) on the development of chick embryos
Document source	Siriraj Medical Journal (2018), Vol. 70, No. 5, pp. 419 28
Short description of literature article	This study was conducted to investigate the teratogenic effects of glyphosate-based herbicide (GBH) by using chick embryo as an animal model. The equal volume of 0.1 mL of 0.01%, 0.05%, 0.3%, and 0.5% w/v glyphosate solution was injected into yolk sacs of fertilized White Leghorn eggs at 21 h of incubation and repeated at the volume of 0.05 mL on the 3rd day of incubation. The embryos were observed for abnormalities on day 3, 6, and 10 of incubation.
Short description of findings	The results showed that the mortality percentages increased as the concentration of glyphosate increased. Day 3 chick embryos showed retardation of development and several abnormalities, for instance, the irregular shape of the brain vesicles with an opening of anterior neuropore, small eye primordia with the optic cup and lens vesicle retardation, looser of the heart looping with dilated lumen, lesser number of branchial arches, absent of limb bud or tail fold. Day 6 chick embryos showed severe retardation of several organs. Microphthalmia, anophthalmia, ectopia cordis and ectopic viscerae were observed in day 6 chick embryos. On day 10, most embryos died earlier and living embryos showed normal external features but delayed ossifications which were significantly different from the control ($P < 0.05$).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Roundup herbicide, a commercial product of Monsanto Company, Malaysia, was studied. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.
RMS comments and conclusion	There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. The exposure method (injection into embryos) is not an environmentally-realistic exposure pathway and hence this study is not considered relevant for the risk assessment. Moreover, due to high

	embryo mortality, it is likely that the developmental effects were a result of systemic toxicity, and hence the results are not relevant for assessment of ED properties.

Data point	CA 9
Report author	Szemeredy G. *et al.*
Report year	2016
Report title	Toxicity test of individual and combined toxic effects of herbicide GLIALKA STAR and lead acetate on chicken embryos. Original Title: GLIALKA STAR gyomirto szer es az olom acetat egyedi es interakcios toxicitasanak vizsgalata madarembriokban.
Document source	Novenyvdelem (2016), Vol. 52, No. 10, pp. 483 487
Short description of literature article	The aim of this study was to determine the individual and combined toxic effects of GLIALKA STAR herbicide (glyphosate 360 g/L) and lead acetate on the development of chicken embryos. On the first day of incubation, chicken eggs were injected with 0.1 mL of the test material. The applied concentrations of lead acetate were 0.01%, 0.1% and of herbicide GLIALKA STAR was 2%. The chicken embryos were examined on day 19 by the following: rate of embryo mortality, body mass, and type of developmental anomalies by macroscopic examination. The body weight was evaluated statistically by the one way ANOVA with Tukey and Dunnett post hoc tests, the embryo mortality and the developmental anomalies were analysed by Fisher test.
Short description of findings	The study revealed that the combined administration of lead acetate and glyphosate containing herbicide formulation (GLIALKA STAR) caused a significant reduction in body weight of embryos and increased the rate of embryonic mortality in both of the applied group. The joint toxic effect of lead acetate and GLIALKA STAR is an additive effect compared to the individual toxicity of the test materials.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested via injection to chicken embryos. This dosing approach does not represent a typical route of exposure. The tested formulation GLIALKA STAR is not the EU representative formulation for the glyphosate EU renewal. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.
RMS comments and conclusion	There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable to the representative formulation. However, we agree that the exposure pathway (injection into embryos) is not representative of field conditions and hence this study is not relevant for the risk assessment. Further, due to the significant embryo mortality it seems that the treatment levels were above the MTC (maximum tolerable

	concentration), and therefore the results are also less useful for the ED assessment.

Data point	CA 9
Report author	Imre P. *et al.*
Report year	2018
Report title	Toxicity test of individual and combined toxic effects of herbicide Amega and copper-sulphate on pheasant embryos. Original title: Amega gyomirto szer es a rez-szulfat egyedi es egyuttes mereghatasanak vizsgalata facanembriokban.
Document source	Novenyvdelem (2018), Vol. 54, No. 11, pp. 476-482
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

The authors have studied the single and combined toxicity of the Amega herbicide and copper sulphate (the latter modelling the environmental pollution caused by metals) on pheasant embryos. A 0.01% copper sulphate solution and a 2% emulsion of Amega (360 g/L glyphosate) were used as test substances. The immersion treatment was done before hatching began and the pheasant eggs were processed on the 21st day of hatching. Embryonic body weights were measured and recorded during necropsy processing, and the number of deaths and incidence of macroscopic malformations were recorded as well. Single treatment with 0.01% copper sulphate solution resulted in a decrease in pheasant embryo body weight compared to the control group, but the difference was not statistically significant. Compared to the control group, there was a significant difference in body weight after the treatment with Amega 2% emulsion and the combined use of the test substances, respectively. The incidence of embryonic mortality and growth anomalies increased slightly for both the single and combined treatment compared to the control group, but the difference was not statistically significant. The single toxicity of the 0.01% copper sulphate solution as well as of the 2% Amega herbicide used in the test was slightly noticed in pheasant embryos growing in the egg. No teratogenic effects could be demonstrated. With the combined use of the two test substances - copper (with showed low embryotoxicity when used alone) and Amega (a well-known herbicide in plant protection) - the embryotoxic effects slightly increased, but this phenomenon did not turn out to have an additive character.

Materials and methods

For modeling the partial environmental load of copper, a 0.01% copper sulfate solution was used based on the study of Fejes (2005). Chicken [*Gallus g. domesticus*] embryos were treated with copper sulfate solutions of 1.0%; 0.1%; 0.01% and 0.001%. Since the copper sulfate solution with the lowest embryotoxic effect proved to be of 0.01%, it was decided to use this concentration in the study as well. Amega herbicide (supplied by Nufarm Hungária Kft., Hungary) containing 360 g/L glyphosate in both single and combined toxicity studies was used (the highest spray concentration was 2%). Ten hours before hatching, eggs were preheated in a 20-22 °C room. The hatching period of eggs is usually 24 days, incubation is done in 2 stages: setting lasts 20 days and hatching lasts 4 days. A temperature of 37.8 °C and 48.0-51.0% relative humidity during the setting period, and 37.5 °C and 65-80% relative humidity during the hatching period was set with a continuous turning of the eggs. Pheasant eggs were

treated just before hatching began. The eggs were divided into random groups (n = 55/group), taking care to form homologous groups based on weight and size, followed by 30 minutes immersion in the test materials: 2% Amega emulsion at 37 °C and 0.01% copper sulfate solution or a combination thereof. Following the immersion treatment, hatching was initiated. The pheasant eggs were opened 3 days before the expected date of hatching, i.e. on the 21st day. During the necropsy, the weight values of live embryos were recorded, the type and frequency of macroscopic malformations examined, and the number of dead embryos and the day (number) of embryonic development when the death occurred determined. Body weight distribution of live embryos was graphically verified by Comparison-Quantile Plot and statistically evaluated by single-variable variance analysis (One Way ANOVA). For pairwise comparison, the TUKEY HSD test was used. Fisher's exact test was used for the biometric processing of growth anomalies and embryonic mortality data. The minimum level of significance was $p < 0.05$ for statistical evaluation.

Results

Control

Embryonic body weight change was 11.20 ± 1.09 g in the mean of 38 live embryos in the control group. (Fig. 1) with 5 dead embryos registered on the 21st day of processing with a ratio of 11.63% relative to fertile eggs. In addition, 2 embryos with abnormal paw (5.26%) were recorded among living embryos, which allowed the group to be used as a reference (Table 1).

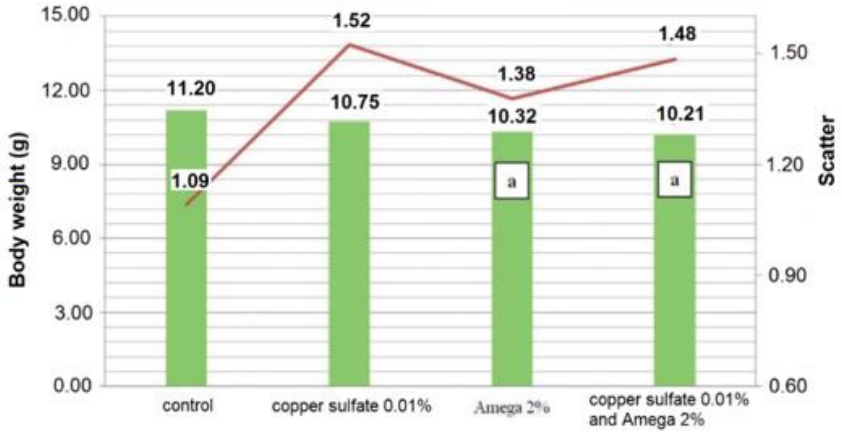


Figure 1 (from Imre P. *et al.* 2018, Novenyvdelem, Vol. 54, No. 11, pp. 476-482). Changes in average body weight (g) of pheasant embryos during the birds teratology immersion test with the single and combined use of copper sulfate and glyphosate-based Amega herbicide. aSignificant difference compared to the control group ($p < 0.05$).

Table 1 (from Imre P. *et al.* 2018, Novenyvdelem, Vol. 54, No. 11, pp. 476-482): Changes in the number and rate of malformations and mortality in pheasant embryos treated with 0.01% copper sulfate and 2% glyphosate-based Amega herbicide.

Treatment	Number of embryos with abnormal growth / number of live embryos	Dead / total fertile eggs (pieces)	Proportion of embryos with abnormal growth (%)	Proportion (%) of dead embryos	Proportion (%) of live embryos
Control	2/38	5/43	5.26	11.63	88.37
Copper sulfate 0.01%	4/36	6/42	11.11	14.29	85.71
Amega 2%	5/34	8/42	14.71	19.05	80.95
Copper sulfate 0.01% + Amega 2%	6/33	10/43	18.18	23.26	76.74

Copper sulphate

The average weight of embryos in the group single treated with 0.01% solution of copper sulfate was 10.75±1.52 g. The weight of embryos in the group single treated with copper sulfate solution was lower compared to the weight of the control group embryos (mean 11.20±1.09 g), but this was not statistically

significant (Fig. 1). In the study of the single toxicity of copper sulfate, 6 dead embryos (14.29%) were registered in 42 fertile eggs, which is a slight difference compared to the control group (5; 11.63%). The proportion of live embryos was 85.71% (Table 1) compared to the control group, therefore the difference was not significant. In a single heavy metal loading test with 0.01% solution of copper sulfate, 4 out of 36 live embryos (11.11%) showed malformations (Table 1), and one case of cephalocele and 3 cases of abnormal foot position were also observed.

Amega
During the single toxicity test of a 2% emulsion of glyphosate-based Amega herbicide, the mean weight of the members of the treated group was 10.32±1.38 g. The measured body weight values were significantly ($p < 0.05$) different from those of the control group (mean 11.20±1.09 g) (Fig. 1).When processing the 2% Amega herbicide single toxicity test results, the number of dead embryos was 8, the rate of embryo lethality was 19.05%, and the proportion of live embryos was 80.95% (Table 1).When using the glyphosate-based Amega herbicide alone, 5 out of the total number of live embryos (14.71%) showed macroscopic malformations (Table 1), i.e. leg deformity.

Copper sulphate and Amega
After a combined treatment with 0.01% copper sulfate solution and 2% glyphosate-based Amega herbicide emulsion, the interaction toxicity test resulted in a mean body weight of 10.21±1.48 g. Body weight values in the group were significantly lower ($p <0.05$) than the mean values of the control group (11.20±1.09 g) (Fig. 1). During the interaction toxicity study with test substances, the number of dead embryos increased to 10 (23.26%), which is twice the value recorded in the control group (5 dead embryos, i.e. 11.63%). (Table 1). Higher rates of embryo mortality compared to the control group were not statistically proven when applying single and combined treatments with copper sulfate and Amega herbicide, but from biological angle, more embryo lethality, compared to the control group, occurred probably due to the treatment with test substances.

In a combined toxicity study with 0.01% copper sulphate solution and 2% Amega emulsion, the number of embryos in the group showing abnormal growth increased to 6 (18.18%). (Table 1). Regarding the types of growth anomalies, abnormal foot posture was diagnosed in all 6 cases. Compared to the control group, the incidence of malformations in living embryos increased during single and combined toxicity tests, but the difference was not statistically significant, but from biologic angle was probably due to the effects of the treatment with the test substances. During the statistical evaluation of macroscopic malformations, there was no significant difference between the single and combined treatments and the control group and between the single and combination treatment. The incidence of malformations in single and combined toxicity tests with 0.01% copper sulfate solution and Amega 2% emulsion was sporadic.

Conclusion

During the studies, the mean weight of the 38 live embryos in the control group was 11.20±1.09 g. Two embryos with macroscopic malformations were found in the group, which is natural, since embryonic malformations are much more common in poultry than in mammals. The main reason for this is that eggs are exposed to environmental effects much more directly than mammals. When performing the single toxicity test, 0.01% copper sulfate solution was slightly embryotoxic, shown by non-significant weight loss due to the treatment; embryonic mortality, which was statistically non-significant, but still higher than in the control group; and sporadic growth anomalies.

The single toxicity test with Amega 2% emulsion showed embryotoxic effects, which shown as significant ($p < 0.05$) weight loss in the treated group on the 21st day of the treatment compared to the control group. Single treatment increased the number of growth anomalies and the degree of embryo lethality, but the teratogenic effect of the herbicide was not statistically proven.

Combined treatment with 0.01% copper sulfate solution and 2% Amega emulsion resulted in a significant ($p < 0.05$) reduction in the weight of the treated embryos compared to the control group. The rate of embryo lethality and malformations observed during the tests was not statistically significant

compared to control group. The teratogenic effect of the herbicide and heavy metal included in the experiment could not be demonstrated.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The formulation tested (Amega) is not the representative formulation for the glyphosate EU renewal. Thus the paper is not relevant for the renewal.
Partly, the observations are caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity).

Assessment and conclusion by RMS:

The RMS does not agree with the applicant's reasoning of non-relevance of this study.
There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation.

The exposure pathway (immersion in glyphosate solution) is rather extreme and unlikely to occur in the field, and thus the RMS considers this study to be not relevant.

A significant reduction in body weight was reported for embryos exposed to 2% Amega. LOEC for body weight is thus 7.2 g a.i./L (2% of 360 g/L). NOEC for both embryo malformations and mortality is determined to be 7.2 g a.i./L.

The RMS considers this study as 'reliable with restrictions', since no analytical confirmation of test concentrations was performed.

Data point	CA 9
Report author	Winnick B. and Dzialowski E. M.
Report year	2013
Report title	The effects of glyphosate based herbicides on chick embryo morphology during development
Document source	FASEB Journal, (2013) Vol. 27, Supp. 1. Meeting Abstracts. Abstract Number: 874.12 Meeting Info: Experimental Biology 2013, Boston, United States, April 20 24, 2013
Short description of literature article	Glyphosate based herbicides are among the most widely used herbicides in the world. The purpose of this study was to determine developmental toxicity of glyphosate, the active ingredient in the common herbicide Roundup, on developing chicken embryos. Few studies have examined the toxic effects of glyphosate alone versus the full compound formulations of Roundup, which include adjuvants and surfactants. Adjuvants and surfactants are added to aid in solubility and absorption of glyphosate, possibly by making it less water soluble or lipophilic. In this study, embryos were exposed on embryonic day 6 to 2% and 1% Roundup and 1% and 2% glyphosate solutions. Injections were made directly into the air cell of the egg. On embryonic day 18, the embryos were sacrificed for morphometric

	measurements including: wet embryo mass, dry embryo mass, yolk mass, liver mass, heart mass, tibiotarsus length and beak length. Internal and external pipping was documented in eggs after embryonic day 18. Embryos treated with 2% glyphosate and 1% Roundup showed significant reductions in wet and dry embryo mass, heart mass, liver mass, tibiotarsus length and beak length. Yolk mass was also significantly smaller in the Roundup animals. Mortality was also documented up to the time of external pipping. Embryos treated with 2% and 1% Roundup showed increased rates of mortality as compared with controls and active ingredient treatments.
Short description of findings	Direct injection of a glyphosate based herbicide to developing chicken embryos resulted in increased mortality and changes in morphometric measurements.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: This is a conference abstract only. Direct injection of glyphosate based herbicide into fertilized chicken eggs is not a relevant route of exposure, in this invalidated test system.
RMS comments and conclusion	The RMS agrees with the applicant's justification. The exposure regimen is not considered comparable to realistic exposure in the field.

2. Amphibians

Data point	CA 8.1.4
Report author	Gungordu A.
Report year	2013
Report title	Comparative toxicity of methidathion and glyphosate on early life stages of three amphibian species: *Pelophylax ridibundus, Pseudepidalea viridis, and Xenopus laevis*.
Document source	Aquatic toxicology (2013), Vol. 140-141, pp. 220-228
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The toxic effect of glyphosate was evaluated on early developmental stages of three anurans, two natural species (*Pelophylax ridibundus, Pseudepidalea viridis*) and one laboratory species (*Xenopus laevis*). The test comprised 4 replicates, containing 20 tadpoles each, using a total of 80 tadpoles. The test medium was changed every 24 hours during the 96 hours test period.

The 96-h LC_{50} values for glyphosate were determined as 25.7 mg a.s./L for *P. viridis*, 27.4 mg a.s./L for *P. ridibundus*, and 15.3 mg a.s./L for *X. laevis* tadpoles. The most sensitive species exposed to glyphosate was *X. laevis*.

Materials and methods

Chemicals and reagents
The formulation Roundup was purchased from a local agrochemical store. The formulation contains isopropylamine salt of glyphosate at 441 g/L and the surfactant polyoxyethyleneamine.

Test organisms
The FETAX solution was used in all breeding tanks for all test species. Test media of controls and all treatment groups were prepared using FETAX solution. It was composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot * 2H_2O$, and 75 mg $MgSO_4$/L distilled water. The mean values of pH, conductivity, and dissolved oxygen in the used FETAX solution were determined to be 7.5 ± 0.03, 1.51 ± 0.05 mS/cm and 8.2 ± 0.25 mg/L.

X. laevis tadpoles were obtained from adult frog pairs that were maintained in a frog colony in our laboratory. Fertilized *X. laevis* eggs were selected under a stereomicroscope, and normal developing embryos were kept in well-aerated FETAX solution until embryos reached the tadpole stage 46. *P. viridis* and *P. ridibundus* eggs were collected during April and May 2011 from a creek near the Campus of Inonu University. All the eggs were collected in one night after spawning, so they likely had minimal exposure to environmental contaminants and hatched in well aerated aquaria within FETAX solution at 23 °C and a 12:12-h light-dark cycle. Four day-old tadpoles that were obtained from these eggs were used as test organisms in this study. All tadpoles used for the assays were in the same developmental period. Stage 46 tadpoles of *X. laevis* were equal to stage 24 of *P. ridibundus* and *P. viridis*. The tadpoles of control groups of each species were maintained in FETAX solution. All dilutions of pesticides were prepared daily in FETAX solution. Tadpoles were exposed to pesticide solutions at 23 °C (±1 °C) with a 12:12-h light:dark photoperiod in semi-static test conditions.

Bioassays
For the toxicity tests, groups containing 20 tadpoles were randomly placed into covered polycarbonate dishes with 50 mL of different concentrations of glyphosate (18.8-51.2 mg a.s./L for P. *viridis* and *P. ridibundus*, 12.8-56.2 for *X. laevis*) solutions. All groups were tested with 4 replicate dishes, containing 20 tadpoles each, using a total of 80 tadpoles. Test solutions were changed every 24 h during the 4-day test periods. The dead tadpoles were removed, and the incidences were recorded. At the end of the experiment, median lethal concentrations (LC_{50}) were determined for 24-, 48-, 72-, and 96-h exposure periods.

Statistical analysis
The LC_{50} values were calculated by probit analysis using SPSS Version 15.0 software (SPSS Inc., USA). Normality was tested with the Shapiro-Wilk test and Levene's test was used to test for homogeneity of variances. One-way analysis of variance (ANOVA) was used, followed by pairwise comparisons of treatment/control grouped data using independent sample t-test. Significance was designated at $p < 0.05$, $p < 0.01$, and $p < 0.001$ for all data analyzed.

Results

The 96-h LC_{50} values of Roundup were determined to be 25.7 mg of a.s./L for *P. viridis*, 27.4 mg a.s./L for *P. ridibundus*, and 15.3 mg a.s./L for *X. laevis*.

Table 1 (from Gungordu A. 2013, Aquat. Toxicol, Vol. 140-141, pp. 220-228): The 96h-LC_{50} values of glyphosate (Roundup) on tadpoles of three frog species.

	Glyphosate					
	Test range (mg AI/L)	No. tested cons.	LC_{50} (mg AI/L)			
			24 h	48 h	72 h	96 h
P. viridis[a]	21.9–51.2	12	[b]	34.6(34.1–35.1)	30.9(30.5–31.2)	26.7(26.4–27.1)
P. viridis	18.8–51.2	14	[b]	35.9(33.8–38.3)	28.8(28.5–29.1)	25.7(25.3–26.0)
P. ridibundus	18.8–51.2	14	36.7(35.9–37.5)	32.9(31.8–34.9)	30.0(29.0–31.0)	27.4(26.4–28.5)
X. laevis	12.8–56.2	11	33.6(32.0–35.4)	22.0(21.6–22.4)	17.6(17.3–17.9)	15.3(13.3–16.9)

[a] The results of a preliminary study.
[b] Concentrations–response relation is insufficient for determination LC_{50} value.

The 96-h LC_{50} values of glyphosate (Roundup) ranged from 15.3 to 27.4 mg a.s./L for *P. ridibundus*, *P. viridis*, and *X. laevis*. The most sensitive species to toxicity of the tested pesticide was *X. laevis* according to the calculated 96-h LC_{50} values.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Acute endpoints for amphibians are not a data requirement for the EU ecotoxicological regulatory risk assessment, as there are no recognised guidelines.

Further points for clarification:
The glyphosate formulation used is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). The product was stated as being Roundup and that it contained polyethoxylated ethylene amine (POEA) surfactant, which is not permitted for use in formulated herbicidal products in the EU. Therefore, the findings of this paper should be treated with a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The results are not considered relevant for the assessment of the representative product since the tested formulation includes substances that are not allowed in the EU (Regulation (EU) 2016/1313 and/or DRAFT Regulation amending Annex III of Regulation (EC) 1107/2009).

Data point	CA 8.1.5
Report author	Paganelli A. *et al.*
Report year	2010
Report title	Glyphosate-based herbicides produce teratogenic effects on vertebrates by impairing retinoic acid signaling
Document source	Chemical research in toxicology (2010), Vol. 23, No. 10, pp. 1586-1595
Guidelines followed in study	None

Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

An embryological approach was used to explore the effects of low doses of glyphosate in development of *Xenopus laevis* embryos. *Xenopus* embryos were incubated with 1/5000 dilution of a commercial glyphosate-based herbicide (GBH) (equivalent to 430 µM of glyphosate).

The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior-posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Therefore, it was concluded that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in *Xenopus* embryos and co-treatment with a RA antagonist rescued the teratogenic effects of the GBH. Consequently, the phenotypes produced by the glyphosate-based herbicide are mainly a consequence of the increase of endogenous retinoid activity. This is consistent with the decrease of Sonic hedgehog (*Shh*) signalling from the embryonic dorsal midline, with the inhibition of *otx2* expression and with the disruption of cephalic neural crest development.

Materials and methods

Test item
Roundup Classic (Monsanto), purity: 48 % w/v glyphosate salt. Glyphosate (Sigma 337757).

Test organism
Xenopus laevis embryos were obtained by in vitro fertilization, incubated in 0.1× modified Barth's saline (MBS) and staged according to Nieuwkoop and Faber. Embryos were tested from the 2-cell stage.

Test design
Tests were performed at the following dilutions series of the formulation: 1/3000, 1/4000, and 1/5000 prepared in 0.1× modified Barth's saline. For rescue experiments 0.5 or 1 µM Ro-415253 was added at stage 9. Cyclopamine (Sigma C4116) was used at 100 µM concentration in 0.1× modified Barth's saline and was applied from the 2-cell stage until fixation. Embryos were fixed in MEMFA when sibling controls reached the desired stage.

Xenopus embryo injections, whole mount in situ hybridization (WMISH) and cartilage staining
Embryos at the 2-cell stage were injected with 360 or 500 pg of glyphosate (Sigma 337757) per cell into one or both cells at the 2-cell stage. Glyphosate was co-injected with 10 ng of Dextran Oregon Green (DOG, Molecular Probes) to identify the injected side. Embryos were cultured in 0.1 × MBS and fixed in MEMFA when sibling controls reached the desired stage. Wholemount in situ hybridization (WMISH) with digoxigenin-labeled antisense RNA probes was performed without a proteinase K step.

For cartilage visualization, embryos were fixed in MEMFA at stages 45-47, washed with PBS, and stained overnight in 0.04 % Alcian blue, 20 % acetic acid, and 80% ethanol. After extensive washing with ethanol and bleaching with 2 % KOH, embryos were washed with 20 % glycerol and 2 % KOH, and dehydrated through a glycerol/ 2 % KOH series until 80 % glycerol was reached.

Detection of retinoic acid (RA) Activity
Embryos were injected into one cell at the 2-cell stage with 320 pg of the plasmid RAREhplacZ (RAREZ) (and placed immediately in 1/3000, 1/4000, and 1/5000 GBH dilutions). Basal luminescence was detected in un-injected and untreated embryos. The endogenous RA activity was measured in embryos injected with RAREZ and left untreated.

As positive controls, embryos were injected with the RAREZ plasmid and incubated at late blastula stage with 0.5 or 5 μM all-transretinoic acid (RA, Sigma R2625). For rescue experiments, embryos injected with the reporter plasmid were incubated in a 1/4000 dilution of GBH from the 2-cell stage, and when they reached the blastula stage, 1 μM of Ro 41-5253 was added. Finally, when sibling controls reached the neurula stages, all embryos were processed for chemiluminescent quantitation of the reporter activity by using the ß-gal reporter gene assay. Protein extracts and enzymatic reactions were performed. Luminescence was measured on duplicate samples in FlexStation 3 equipment (Molecular Devices), and values were normalized by protein content.

A two-tailed t-test was employed to analyze the significance in the difference of the means. The experiment was repeated three times.

Results

Effects of the glyphosate-based formulation (GBH) and Glyphosate on neural crest markers, rhombomeric patterning, and primary neuron differentiation:
2-cell stage *Xenopus laevis* embryos were exposed at the neurula stage (stage 14-15) to study the effects on neural crest development, rhombomeric patterning, and neuronal differentiation. The neural crest marker *slug* begins its expression early, where neural crest induction takes place. At neurula stage, it was expressed in the neural crest territory. Treated embryos show an important down-regulation of *slug* in the neural crest territory in comparison with that of sibling controls.

To study the effects on hindbrain patterning, the expression of krox-20 which is a zinc finger transcription factor expressed in rhombomeres r3 and r5. The r3 stripe was lost in GBH-treated embryos.

Furthermore, the effects on the primary neurogenesis at the neural plate stage was investigated. *N-tubulin* was normally expressed in differentiated primary neurons organized in three longitudinal domains in the posterior neural plate: medial, intermediate, and lateral, which correspond to motor neurons (m), interneurons (i), and sensory neurons (s), respectively. Treated embryos showed a down-regulation in the three stripes of primary neurons.

To confirm if the effect is specifically due to the active principle of the herbicide and not to adjuvants present in formulations, glyphosate was injected into one cell at the 2-cell stage and *slug, krox-20,* and *N-tubulin* were revealed at stages 14-15, as before. These embryos showed an important downregulation of *slug*, resembling the effects of GBH on this marker at this stage of development. Although *Krox-20* did not completely disappear from r3 as in GBH-treated embryos, the expression clearly decreased in this rhombomere as well as in r5, indicating that glyphosate also alters rhombomeric patterning.

Glyphosate-injected embryos showed that the segregation process clearly affected the injected side, suggesting that the derived cartilages may be affected at later stages during development. When hybridized with N-tubulin, these embryos showed a decrease in the number of primary neurons in the three stripes corresponding to motor neurons, interneurons, and sensory neurons, resembling the effects of GBH treatments, although with milder consequences for this marker.

In conclusion, the effects of GBH-treated and glyphosate-injected embryos represent equivalent phenotypes despite the fact that they are not identical. The adjuvant present in the commercial formulation may explain the differences. Taken together, these results indicate that both GBH and glyphosate impair neuronal differentiation, rhombomeric formation, and the pattern of the neural crest during induction and segregation.

GBH and glyphosate produce head defects and impair the expression of dorsal midline and cephalic markers
Shh expression was dramatically reduced in the dorsal midline at neurula stages, especially in the prechordal mesoderm in GBH-treated embryos. The anterior limit of the *shh* expression domain is moved caudally in treated embryos, in relation to the *pax6* domain.

Embryos incubated with GBH showed a distinct down-regulation of the p*ax6* territory. Moreover, in treated embryos, the *pax6* domain is not divided in the eye field. Therefore, it can be assumed that a down-regulation of shh expression in the prechordal mesoderm together with a diminution of *pax6* expression might underlie defects in the resolution of the retina field and the brain hemispheres in embryos treated with GBH.

Exposed embryos showed a decrease of anterior *shh* expression with concomitant microphtalmy and microcephaly, as revealed by the reduction of the *otx2* domain. Also, there is a pronounced shortening of the A-P axis. In control embryos, the transcription factor sox9 is expressed in the cranial neural crest cells as they populate the pharyngeal arches, the otic placode, the developing eye, the genital ridges, and also the notochord.

Embryos treated with GBH showed reduced eyes and genital ridges, and developed abnormal pharyngeal arches. The migration of neural crest cells to these structures was delayed, as revealed by a more dorsal position.
Similar to embryos treated with GBH, a reduced prechordal *shh* expression was observed accompanied by strong microcephalic and microphtalmic phenotypes.

Taken together, all of these results indicate that GBH as well as glyphosate alone cause cephalic defects that probably result from a reduction of *shh* and *otx2* expression in anterior structures. There was a delay in the migration of cranial neural crest cells in the tailbud stage embryos and the slug expression at earlier stages was inhibited.

GBH and glyphosate disrupt the development of the craniofacial skeleton
To address if the effects seen at neurula and tailbud stages were correlated with craniofacial malformations, embryos treated with GBH and embryos unilaterally or bilaterally injected with glyphosate at the 2-cell stage were allowed to develop up to stage 47 and processed with Alcian Blue staining for skeletal analysis. The gross morphology of GBH-treated embryos revealed an overall reduction of cranial structures and microphthalmy. All affected embryos displayed a reduction of the quadrate and Meckel's cartilages, while the branchial and cerathoyal cartilages were mildly affected.

Unilateral glyphosate injections resulted in a general decrease of Alcian blue staining and in a reduction of the Meckel's and quadrate cartilages on the injected side. In some embryos, the eye practically disappeared from the injected side. Moreover, bilaterally injected embryos exhibited cyclopia, consistent with the loss of *Shh* signaling from the prechordal mesoderm observed at earlier stages.

In summary, these malformations suggest the loss of midline signaling, accompanied by defects in neural crest migration (or increased apoptosis) with aberrant development of mandibular and maxillary structures.

Phenotype induced by GBH is at least mediated by changes in retinoic acid (RA) signaling
GBH treatment significantly increased the level of RA signaling in the embryo in a concentration-dependent manner (Fig. 1). Importantly, the RA receptor antagonist Ro rescued the effect of GBH since the level of the RA output, as measured by the reporter assay, was not significantly different from that in RAREZ-injected, untreated controls. Together, these observations strongly suggest that GBH increases endogenous retinoid activity.

Control embryos showed an expression of *otx2* in the forebrain, midbrain, and optic vesicle, while *shh* transcripts are distributed along the embryonic dorsal midline. Embryos treated continuously with GBH showed a downregulation of *shh* and *otx2*, reduced head structures, and shortened A-P axis.

It was concluded that the ability of Ro treatment to rescue the teratogenic effect of the GBH supports the idea that RA activity is elevated in GBH-treated embryos.

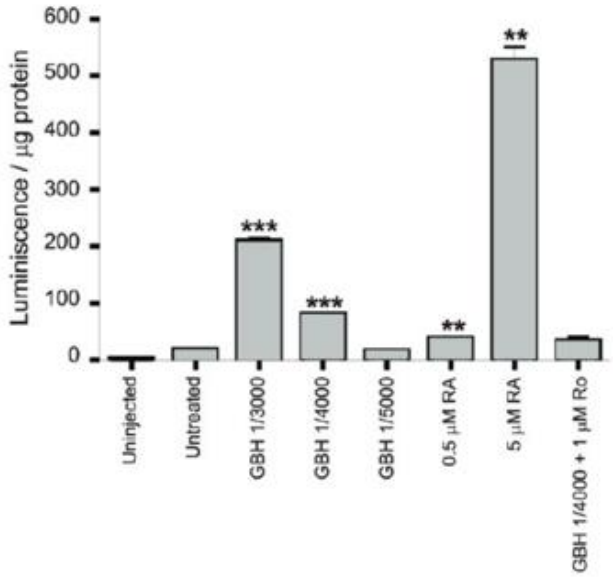


Figure 1 (from Paganelli A. *et al*. 2010, Chem. Res. Toxicol, Vol. 23, No. 10, pp. 1586-1595). Phenotype induced by GBH is mediated by an increase of RA signaling: Analysis of RA activity with the reporter plasmid RAREZ. All embryos were injected with the reporter plasmid RAREZ, except for un-injected controls, and left untreated or were treated as indicated in the figure until stage 14-15, when they were processed. Results are expressed as arbitrary luminescence units per µg of protein. A two-tailed t test was employed to analyze the significance in the difference of the means. ** $p < 0.01$; *** $p < 0.0001$.

The commercial glyphosate-based herbicide (GBH) formulation and glyphosate itself interfere with key molecular mechanisms regulating early development in *Xenopus* embryos, leading to congenital malformations.
Sublethal doses (430 µM of glyphosate in 1/5000 dilutions of GBH) and injections leading to a final concentration of 8 to 12 µM of glyphosate in the injected side of the embryo were sufficient to induce serious disturbances in the expression of *slug* (neural crest marker), *otx2* (gene expressed in retinal and lens components of the eye and telencephalic, diencephalic, and mesencephalic regions) and Sonic hedgehog (*shh*). These molecular phenotypes were correlated with a disruption of developmental mechanisms involving the neural crest, embryonic dorsal midline formation, and cephalic patterning.

Treatments with a GBH increase endogenous retinoic acid activity, as measured by the reporter plasmid RAREZ, and that the GBH-induced phenotypes are rescued by the antiretinoid Ro 41-5253, an antagonist of the retinoic acid receptor.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Study to look at the effect of glyphosate / glyphosate product on the developmental effects of *Xenopus laevis* embryos. Study was conducted in Argentina. Very high concentrations were tested and an unrealistic route of exposure was examined (glyphosate was injected into embryos). In addition, the tested formulation is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). Moreover, no relevant endpoint was generated which can be used in the EU ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Further points of clarification:

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS agrees that injection of glyphosate into embryos is not an exposure route representative for field conditions. However, this method demonstrates the same effects on the early mechanism of morphogenesis as those produced by the glyphosate formulation (that can penetrate the cells directly), consisting of a down-regulation of the *slug* neural crest marker and alteration of rhombomeric patterning.

There is not sufficient information in the study report to demonstrate whether the tested formulation is comparable with the representative formulation.

It is noted that, beside the amphibian species, this study also investigated chicken embryos, yet those results were not mentioned in the summary above. According to the study report, the glyphosate formulation produced similar effects in chicken embryos as for amphibians, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly.

The RMS considers this study less useful for risk assessment purposes due to the exposure regimen (levels and route), while the relevance for ED-assessment is not clear, due to difficulties in comparing the dose with the MTD.

The study has two major limitations, namely lack of analytical verification and suboptimal design of the control group (no real 'procedural' control). The RMS concludes that the study is not relevant and not reliable.

Data point	CA 9
Report author	Berger G. *et al.*
Report year	2018
Report title	How does changing pesticide usage over time affect migrating amphibians: a case study on the use of glyphosate-based herbicides in German agriculture over 20 years.
Document source	Frontiers in Environmental Science (2018), Vol. 6, article 6
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability as provided in the AIR5 dossier (KCA 9) by the applicant; See also RMS analysis in RMS box	Not relevant by full text

Since its introduction in 1974, the use of glyphosate in agriculture has been continuously increasing; however, the application modes of this herbicide have been changing. Therefore, glyphosate-based herbicides can be used as an appropriate indicator for assessing how changes in pesticide application

modes affect wild-living organisms in agricultural landscapes over time. Amphibians that migrate through arable fields may be exposed to the chemicals applied to field crops. Using data on the temporal coincidence of four amphibian populations with glyphosate applications from a three-year investigation in northeast Germany as well as data on the application of glyphosate to field crops in German agriculture over 20 years, the species-specific increasing rates of coincidence likelihoods during this period was estimated. The overall consumption of glyphosate used in German agriculture between 1992 and 2012 increased by a factor of 5.7, while the species-specific coincidence likelihood increased from 2.2 to 6.1, respectively. These results reveal the highest increases in coincidence for both adult and juvenile great crested newt (*Triturus cristatus*) and fire-bellied toad (*Bombina bombina*). Adults and juveniles of moor frog (*Rana arvalis*) and adults of spadefoot toad (*Pelobates fuscus*) were subjected to moderate increases, with rates ranging from 3.2 to 3.6; in contrast, juvenile individuals of *P. fuscus* showed small increases.

Materials and methods

This project was funded by the Umweltbundesamt, Germany under the award number FKZ: 3709 65 421.

Reference Year and Investigation Periods

Annual crop production and glyphosate (GLY) sales for agriculture varied over the tenure of 20 years (i.e., 1992–2012). Therefore, three average values were considered for this period, the cultivation of arable crops × 1000 ha, which is abbreviated in this paper as Tha, and the GLY sales (t) for the periods of 1991–1993, 2001–2003, and 2011–2013; these data were used as a reference. These average values are referred to as investigation year 1992, 2002, and 2012, respectively. Further, based on a field survey that covered a wide range of agricultural farm situations throughout Germany and was valid for the reference year 2009, the relative share of crop area treated with the three GLY application modes was calculated. The authors supported their calculated data by comparing them with the GLY sales and consumption in German agriculture during this reference year. All extrapolations and investigations of this work are linked to this reference year.

Glyphosate Sale and Usage in German Agriculture from 1988 to 2013

Statistical data on the sale and usage of GLY in German agriculture were available, though data were of various qualities. Data on GLY usage from 2009 onwards, the consumption between 1995 and 2008 and on the sale of organophosphorus herbicides, were derived by analyzing the annual reports of BVL (Bundesamt für Verbraucherschutz und Landwirtschaft), which is the regulating authority for pesticides in Germany. The annual values of the consumption of GLY from 1988 to 1994 were derived by applying the assessed trend function for the annual increase ($yc = 166.45x + 129.7$; for 1988: $x = 1$, and for 1994: $x = 7$), which was revealed by pre-analyses. Estimations were verified by visually analyzing the extrapolated values on the chart. The amount of GLY sold to professional users during the investigation years are based on the average sales from three consecutive years. Of the total estimated sales, 90% was used by farmers and the remaining 10% was used by other professionals belonging to horticulture, viniculture, railway companies and municipalities.

Expert Estimation and Validation of Glyphosate Usage Per Application Scheme and Crop

The usage schemes of GLY in agriculture for the investigation years 1992, 2002, and 2012 were jointly assessed by consensus of four experts with knowledge of GLY use in agriculture. First, the experts listed the changes in 1992, 2002, and 2012 as compared to the reference year 2009. Second, these identified changes were translated into relative numbers. Third, based on the numbers relative to 2009, the proportion of area where GLY was applied was calculated, considering all application schemes and crops for the identified study years. Multiplying this value by the total cultivation area of crops, the area of crops treated with GLY was derived for each investigation year and included the application modes. Based on the application rate of GLY per hectare, and the application modes for all crops, the amount of GLY applied in the investigation years was calculated, as well as the total amount of GLY usage in German agriculture for each investigation year and then compared to the corresponding statistical data on GLY sales and consumption in German agriculture. When the deviance between the calculated values

and the real values was less than 5%, the values were considered to be satisfactory; in contrast, higher deviations were used to iteratively adapt the estimates that the experts felt less confident about.

Field Data on the Temporal Coincidence of Amphibians with Glyphosate Applications to Crops
The quantitative field data available for the temporal coincidence of amphibian populations with the application of GLY on arable fields were provided by a field survey carried out between 2006 and 2008 in a study area located 50 km east of Berlin, Germany. This landscape has intensive agriculture use and is pond rich, so four typically occurring amphibian species [fire-bellied toad (*Bombina bombina*, Linnaeus, 1761), moor frog (*Rana arvalis*, Nilsson, 1842), spadefoot toad (*Pelobates fuscus*, Laurenti, 1768) and northern crested newt (*Triturus cristatus*, Laurenti, 1768)] were analyzed by using fence trapping during the annual migration periods. These amphibian species cover a wide range of different migration periods. Forty-nine drift fences, which consisted of 26 open, 10-m-long, cross-shaped fences, and 23 enclosures were installed between field machinery tramlines. The cross-shaped fences were regularly distributed in a 400 × 400 m grid to record amphibian migration activity in fields, and they encircled biotopes (i.e., wood lots, small water bodies) located at the edges of fields or completely within fields. Depending on the direction of migration, either the inner or outer traps were analyzed. Captured individuals were released 10–15m from the opposite site of the fence. Three different application modes (e.g., presowing or pre-emerging application in spring, siccation in summer, and pre-harvesting and stubble management in late summer/autumn prior to crop sowing) and six major field crops (maize, triticale, winter barley, winter rape, winter rye and winter wheat) were included; additionally, the four amphibian species listed above and their age-specific coincidence values for two DT50 values of GLY (i.e., 12 and 47 days) were used separately. These values allowed the derivation of important indicators for the co-occurrence and potential exposure of amphibian populations. The DT_{50} values for GLY must serve as proxies for GBH. Coincidence values for each GLY application scheme and for each crop as averages of the two DT_{50} levels were calculated and applied.

Calculating the Species and Age-Class Specific Changes of Coincidence Values
Changes in the coincidence likelihood between amphibian populations and the application of GLY to field crops depend on (a) the increase in GLY application area per application scheme over time and (b) the species and their age-level-specific share of the amphibian populations. To find a species and age-specific indicator value suitable for explaining changes over years, the coincidence values and the GLY application area were multiplied, which enabled the calculation of the coincidence likelihood. The ratio of the obtained products indicates the likelihood of an increase in coincidence between periods. The analyses were performed for the four amphibian species, their two age levels, and the two migration periods (i.e., from and to ponds).

Results

Overall Glyphosate Sales and Consumption in German Agriculture Over the Last 20 Years
In Germany, GLY usage by professional applicators (e.g., agriculture, horticulture, railway companies, municipalities) increased from 956 t of active substance in 1992 to 5415 t of active substance in 2012. Thus, the consumption in 2012 was 5.7 times higher than the consumption 20 years ago. In 2002, approximately 3740 t GLY was applied, corresponding to a 3.9-fold increase compared to 1992 and a 1.5-fold increase from 2002 to 2012. In terms of agricultural use, it is assumed that 90% of the total consumption was by professionals (860, 3.365 and 4.873 t, respectively), leading to the same increase in values between periods.

Expert Estimations on the Relative Changes of Glyphosate Application Modes to Arable Crops Over 20 Years
The factors relative to the GLY application in 2009, as estimated by experts, varied between the crops and periods (Fig. 1). Except for maize, which had a factor of 0.5, the pre-sowing applications to all other crops in 1992 were estimated to be approximately 0.1 of the application values of 2009. In both 2002 and 2012, the application values for this crop surpassed the application value of 2009. In 2002, the other range of crop factors (relative to 2009) varied from 0.7 to 0.8, and this value was 1.0 in 2012. For pre-sowing application, compared to the values from 2009, an additional increase of 1.2 for winter wheat and an increase of 1.1 for both oilseed rape and corn, was estimated.

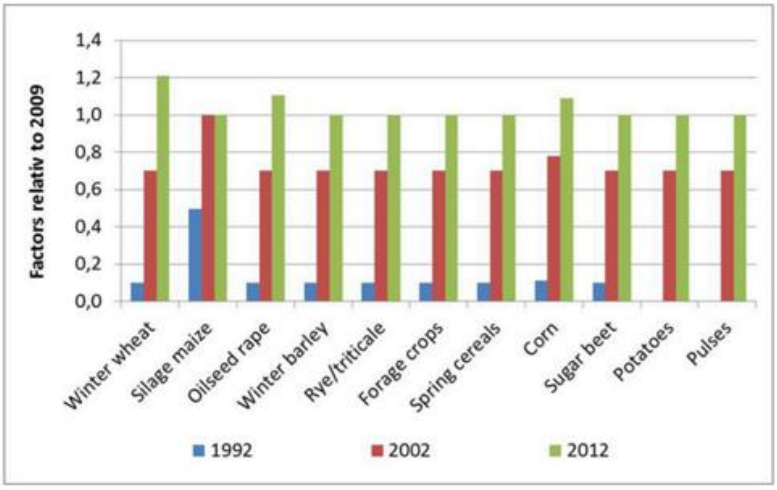


Figure 1 (from Berger G. *et al*. 2018, Front. Environ. Sci., Vol. 6): Expert estimations of the relative deviances from the reference year 2009 on the amount of GLY applied in pre-sowing applications of GBH to arable crops in the three investigation years.

Calculated Area of Glyphosate Applied on Arable Land and GLY Consumption in German Agriculture
The total area of GLY application in German agriculture, including grassland (not shown), was ≈740 Tha in 1992; ≈2.930 Tha in 2002; and ≈4.250 Tha in 2012. Based on the area of GLY application and the estimated application rates per crop, the consumption of GLY in agriculture was 845 t, 3.340 t, and 4.865 t for 1992, 2002, and 2012, respectively. These values differed between −0.2 and −1.7% from the statistically grounded sale of GLY to agricultural buyers. The areas with most of the 6 main crops, i.e., winter wheat (wwt), silage maize (mze), oilseed rape (wra), winter barley (wbl) and winter rye/triticale (wry/trc), considered for amphibian coincidence analyses increased over 20 years. The increase from 1992 to 2012 ranged from 1.3 to 1.6, though the value for winter barley fell outside this range. The area of maize increased the most between 2002 and 2012, with a rate of 1.8 during this 10-year period. The application area of these 6 crops increased from 663 Tha in 1992 to 2286 Tha in 2002 and to 3461 Tha in 2012, encompassing between 81 and 94% of the total area where GLY was applied on arable land. The GLY application area increased from by factor 5.2 between 1992 and 2012, by a factor of 3.4 between 1992 and 2002, and by a factor of 1.5 between 2002 and 2012. The GLY application area was largely different between periods and plants (Fig. 2). Wwt, wra, and wbl covered large areas with the steepest increases occurring between 1992 and 2002. This contrasts with the GLY application area of mze, which particularly increased between 2002 and 2012, but never reached the application area of wwt, wra and wbl.

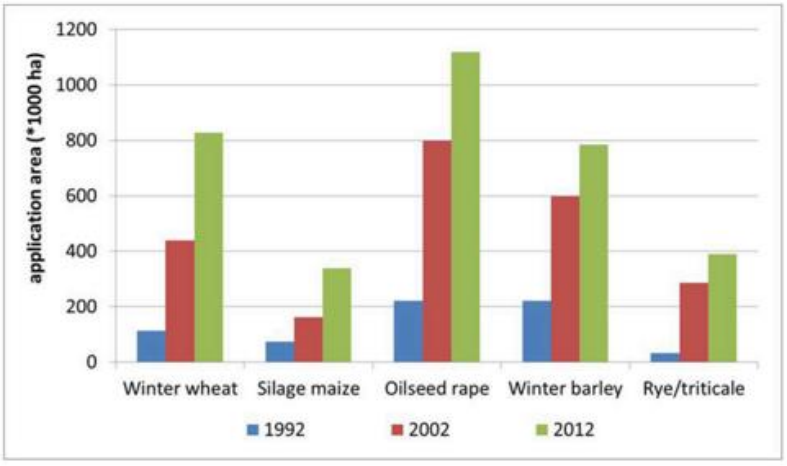


Figure 2 (from Berger G. *et al*. 2018, Front. Environ. Sci., Vol. 6): Total GLY application area for each crop considered for amphibian coincidence during the three investigation periods over 20 years.

Allocation of Glyphosate to Application Modes of Crops Considered for Amphibian Coincidence
Except for pre-harvesting applications for mze, the GLY application areas for all other crops with different application modes increased. The average rate of increase of 9.1 was exceeded or wwt, wra, and rye/trc for all three applications modes. For presowing (ps/pe) and pre-harvesting (ph) in wwt and for ps/pe in wra, a 16-fold increase in rates was found. When comparing the rates of increase between 1992 and 2002 with those between 2002 and 2012, higher values were mostly found during the first period.

As shown for ps/pe and ph in wry/trc, the values were as much as 8-fold higher. With respect to the size of application area, stubble application (sa) of wra was always ranked first during the entire 20-year period, though ranks 2 through 4 occasionally changed over time. From 2002 to 2012, the same combinations of crops and application modes in these rank positions were found, albeit in different orders. In 1992, ps/pe in mze was in rank 4, and ph in wbl was in rank 2. Both lost these ranked positions in following years.

Amphibian-Specific Increase of Coincidence Likelihood
The rise of GLY in agriculture during the 20-year period led to increasing rates of coincidence likelihood of amphibians from 2.2 to 6.1 (Table 1). The amphibian coincidence likelihood increased by an average of a factor of 4.1 between 1992 and 2012, 2.6 between 1992 and 2002, and 1.5 between 2002 and 2012. The coincidence likelihood rates of increase for adults of all four species migrating to ponds were about one third lower than the average coincidence likelihoods observed between 1992 and 2002. The same was found for adults of *P. fuscus* and for juveniles of *R. arvalis* and *P. fuscus* that migrated from ponds. In contrast, adults and juveniles of *T. cristatus* and *B. bombina* largely exceeded the average increases. Their coincidence likelihoods were about one-half higher than the averages and almost twice as high than the other amphibian groups. Between 2002 and 2012, the increases in coincidence likelihoods for the investigated amphibian groups were different than the values from the preceding decade (Table 1). The rate of increase of coincidence likelihoods for adults of all species migrating into ponds and for adults of *P. fuscus* leaving ponds were above average. However, for all juveniles and for adults of *T. cristatus* and *B. bombina*, increases of coincidence likelihoods were found that were equal or lower than the average value.

Table 1 (from Berger G. *et al*. 2018, Front. Environ. Sci., Vol. 6). Cumulative product values and rate of increase according to migration, age and species (bold* indicates values above mean).

Migration	Age	Species	Cumulative product values (GLY application area * coincident population share)			Rate of increase		
		Period	1992	2002	2012	1992–2002	2002–2012	1992–2012
To ponds	Adults	*R. arvalis*	1.5334	2.7762	4.9398	1.8	**1.8***	3.2
		T. cristatus	2.4904	4.5087	8.0225	1.8	**1.8***	3.2
		P. fuscus	1.4154	2.5624	4.5595	1.8	**1.8***	3.2
		B. bombina	4.2799	7.7486	13.7875	1.8	**1.8***	3.2
From ponds	Adults	*T. cristatus*	13.2085	51.5473	80.2051	**3.9***	1.6	**6.1***
		P. fuscus	2.543, 5	4.6048	8.1936	1.8	**1.8***	3.2
		B. bombina	13.9743	52.0846	78.7004	**3.7***	1.5	**5.6***
	Juveniles	*Rana arvalis*	8.0988	20.9970	29.5561	**2.6***	1.4	3.6
		Triturus cristatus	14.8296	60.0357	89.6121	**4.0***	1.5	**6.0***
		Pelobates fuscus	10.5586	17.3677	23.4559	1.6	1.4	2.2
		Bombina bombina	7.3688	30.4893	42.7882	**4.1***	1.4	**5.8***
				Mean		2.6	1.6	4.1

The increases of coincidence likelihoods for the entire investigation period (i.e., 1992–2012) were similar to the changes between 1992 and 2002, though the changes were at various extents (Table 1). With almost a 6-fold rate of increase, the highest values were found for adults and juveniles of *T. cristatus*; in contrast, the coincidence likelihood of juveniles of *P. fuscus* increased only by a factor of 2.2. The increases in coincidence of likelihoods for species and age levels were found to vary between 1.4 and 6.1. For *T. cristatus* and *B. bombina*, the highest increases were recorded between 1992 and 2002, though higher coincidence likelihood increases were found for adults of *P. fuscus* and *R. arvalis*

during the second period. Generally, for the former two species, the rates of increase were lower compared to the others, and, except for *P. fuscus*, there were higher increases for juveniles than for adults over the 20-year period.

Conclusion

Glyphosate usage in German agriculture has changed considerably over the 20-year study period. The analysis investigated how wild-living amphibians present in fields were likely to be increasingly exposed to GLY during this time. For the environmental risk assessment and regulation of plant protection products, the author of this paper advocates considering not only the state and usage of pesticides at the time of their authorization but also the changes in application schemes and extents over time. Thus, the author recommends conducting periodical reassessments of environmental risks not only on the toxicity to organisms but also, on the present and practical usage modes in agriculture that affect potential exposure patterns.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: This paper considers information from multiple sources to assess the impact of herbicides on amphibian populations in Germany over the last 20 years. This is country-specific information that cannot be related to an EU level ecotoxicological regulatory risk assessment for EU Annex I renewal.
Moreover, no formulations tested were specifically mentioned. However, in the introduction of this paper, the author states that "*Glyphosate-based formulations have been shown to be toxic, especially during the aquatic life stages of amphibians, and often the commonly added surfactant POEA (polyethoxylated tallow amine) is mainly responsible for adverse effects…. "Taking the particular risks of POEA into consideration, POEA free glyphosate-based formulations have increasingly been used in German agriculture since 2013….*" Since this paper summarizes the impact of herbicides on amphibian populations from 1992-2012 this statement adds uncertainty concerning the relevance of these findings to the EU renewal of glyphosate. The representative formulation for the glyphosate EU renewal MON 52276 does not contain POEA (POEA surfactants are not permitted for use in formulated herbicidal products in the EU). As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The RMS agrees with the applicant's conclusion that this study is not relevant for the risk assessment of MON 52276 for this evaluation, since there were no specific results on toxicity of glyphosate. Moreover, the RMS agrees that it is likely that some of the products used between 1992-2012 (and included in this overview) contained co-formulants which are no longer allowed within the EU. Possibly, the results may be useful for discussion on risk mitigation options.

Data point	CA 9
Report author	Jayawardena U. A. *et al.*
Report year	2016
Report title	Combined Effects of Pesticides and Trematode Infections on Hourglass Tree Frog *Polypedates cruciger*
Document source	EcoHealth, (2016) Vol. 13, No. 1, pp. 111 22
Short description of literature article	The study aimed to evaluate on the lone and combined effects of exposure to parasitic cercariae (larval stage) of the digenetic trematode, *Acanthostomum burminis*, and a commonly used herbicide glyphosate (Roundup) at ecologically relevant concentrations on the survival, growth, and development of the common hourglass tree frog, *Polypedates cruciger* Blyth 1852.
Short description of findings	There was no evidence of any pesticide induced mortality on cercariae because all the cercariae successfully penetrated each tadpole host regardless of pesticide treatment. In isolation, both cercarial and pesticide exposure significantly decreased frog survival, development, and growth, and increased developmental malformations, such as scoliosis, kyphosis, and also oedema and skin ulcers. The combination of cercariae and pesticide generally posed greater risk to frogs than either factor alone by decreasing survival or growth or increasing time to metamorphosis or malformations. Consistent with mathematical models that suggest that stress should increase the impact of generalist parasites, the weight of the evidence from the field and laboratory suggests that ecologically relevant concentrations of agrochemicals generally increase the threat that trematodes pose to amphibians, highlighting the importance of elucidating interactions between anthropogenic activities and infectious disease in taxa of conservation concern.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Observations is caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity). The tested glyphosate formulation Roundup is not the representative formulation for the glyphosate EU renewal, as it contains POEA as a surfactant system. Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo climatic properties, land uses and agricultural practices, non EU monitoring data, residue definitions differing from EU). No data presented relevant for the EU regulatory ecotoxicological risk assessment. **Further points of clarification:** Although Roundup 360 SL formulation was tested, it does however contain different surfactant system. As the performance / efficacy of herbicidal formulations is dependent on the surfactant system / co formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal. The surfactant system used in the formulation tested in this paper is polyethoxylene amine (POEA) based, whereas the surfactant used in the representative formulation MON 52276 for the glyphosate EU renewal is quaternary ammonium based. POEA surfactant is not permitted in formulated herbicidal products in the EU. For this reason and also since the influence of the POEA surfactant on the achieved

	findings cannot be excluded, the paper is not relevant for the glyphosate EU renewal.
RMS comments and conclusion	The RMS agrees with the applicant's justification; The results are not considered relevant for the assessment of the representative product since the tested formulations include surfactants that are similar to substances that are not allowed in the EU (Regulation (EU) 2016/1313 and/or DRAFT Regulation amending Annex III of Regulation (EC) 1107/2009).

Data point	CA 9
Report author	Mikó Z. *et al.*
Report year	2017
Report title	Age dependent changes in sensitivity to a pesticide in tadpoles of the common toad (*Bufo bufo*)
Document source	Aquatic Toxicology, (2017) Vol. 187, pp. 48 54
Short description of literature article	In this study tadpoles of the common toad (*Bufo bufo*) were exposed to three concentrations (0, 2 and 4 mg a.e./L) of a glyphosate based herbicide Glyphogan Classic during the 1st, 2nd, 3rd, 4th, or 5th period of larval development or during the entire experiment, and measured survival, time until metamorphosis and body mass at metamorphosis to estimate fitness consequences.
Short description of findings	Younger tadpoles were more sensitive to the herbicide in all measured traits than older ones, and this age dependence was especially pronounced at the high herbicide concentration. Furthermore, tadpoles exposed to the herbicide during the entire experiment developed slower than tadpoles exposed only early on, but no similar effect was observed either on body mass or survival. The observed age dependence of sensitivity to herbicides draws attention to the fact that results of toxicity tests obtained for one age class are not necessarily generalizable across ontogeny. Also, the age of test animals has to be considered when planning ecotoxicological studies and interpreting their results. Finally, taking into account the temporal breeding habits of local amphibians when planning pesticide application would be highly favourable: if tadpoles would not get exposed to the herbicide during their most sensitive early development, they would sustain less anthropogenic damage from the efforts of controlling weeds.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by title/abstract: Tested formulation Glyphogan Classic, contains POEA and its composition is not equivalent to the representative formulation for the glyphosate EU renewal, thus the article is not relevant. Further points of clarification: The representative formulation is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl ammonium salt and a quaternary ammonium based surfactant.
RMS comments and conclusion	The RMS agrees with the applicant's justification. The results are not considered relevant for the assessment of the representative product

	since the tested formulation includes surfactants that are similar to substances not allowed in the EU (Regulation (EU) 2016/1313 and/or DRAFT Regulation amending Annex III of Regulation (EC) 1107/2009).

3. Fish

Data point:	CP 10.2.1/007
Report author	Gabriel, U.U. *et al.*
Report year	2010
Report title	Toxicity of roundup (a glyphosate product) to fingerlings of *Clarias gariepinus*
Document No	ISSN: 159 – 3115
Guidelines followed in study	None
Deviations from current test guideline identified by the applicant: See RMS analysis in RMS comment box	*Not applicable*
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability (RMS):	Less relevant but supplementary and reliable with restrictions (supportive data)

Summary

Acute static renewal bioassays were conducted on fingerling and adult of *Clarias gariepinus* (mean weight, 1.22 ± 0.6g; mean total length, 5.25 ± 1.25 cm) using the herbicide, Roundup (glyphosate). In the acute study, fingerlings were exposed in triplicate to 0.0, 14.0, 16.0, 18.0, 20.0, 22.0 and 24.0 mg/l of the herbicide for 96 hours to determine general behavioural responses.

The authors concluded that 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

However, the similarity of the formulations (Roundup vs. MON 52276) is not established, RMS considered this study being less relevant but supplementary. The 96 hour LC50 of Roundup on the fish was 15.88 mg/l (equivalent to approximately 5.7 mg glyphosate acid equivalent/L). Howvere the results were considered unreliable.

Materials and methods

The fingerlings of *C. gariepinus* (mean weight 1.22 ± 0.6g; mean total length 5.25 ± 1.25 cm) were obtained from a private farm, Comsystem, Kpite, Rivers State and transported in 25 litre jerry can to the Wet Laboratory, Department of Fisheries and Aquatic Environment, Rivers State University of Science and Technology, where they were distributed 60 fish per aquarium in four rectangular aquaria filled with 20 litre borehole water (dissolved oxygen, 0.01 ± 0.05 mg/l, pH- 7.5 ± 1.3; conductivity, 410 ± 20.4 µS/cm; total dissolved solid 400 ± 10.25 ppm). They were fed at one percent biomass, half at 0900 and 1600 hours for a week. Cleaning of the tanks and water exchange were done daily. Mortality during acclimation period was less than one percent. Mucus accumulation on the skin as well as gills and skin pigmentation were recorded.

Range finding test and trial runs were done. Twenty litres of each of the following concentrations: 14, 16, 18, 20, 22 and 24 ppm of Roundup containing 360 g/l glyphosate (in the form of 480g/l isopropylamine salt) and a control were prepared in triplicate in glass aquaria. Ten fish was randomly distributed into each of the tanks. The general behaviours, opercular beat frequency, OBF, tail beat frequency, TBF and mortality (%) were recorded at 12, 24, 48, 72 and 96th hour, respectively. The exposure lasted for 96 hours. Data obtained from the experiments were subjected to ANOVA using Statistical Package for the Social Sciences, SPSS version 15 and differences among means were

separated by Duncan Multiple Range test at 0.05%. The dependent variables in the trials (OBF, TBF and cumulative mortality) were regressed on concentration of the toxicant to obtain the regression lines of best fit for predicting the values of the dependent variables with changes in that of the independent with Microsoft Excel®. Correlation analysis was used to determine the degree of association among the dependent and independent variables. Lethal concentrations (LC50) values for the 24, 48, 72 and 96 hour and the median lethal times (MLT50) for the various concentrations of herbicide were done with Probit Analysis. Safe concentration of the herbicide at the various time intervals were obtained by multiplying the lethal concentration by a factor, 0.1. The interaction effects of the behavioural responses (TBF and OBF) with exposure duration and concentrations of the herbicides were presented graphically.

Results

On introduction into the toxicant the fish showed initial hyper-excitability, stress responses such as increased opercular ventilatory rate, dash and erratic swimming and gasping for air within the first two hours. As exposure time increased before death occurred they "hung" on the surface of the solution gulping air, fell steadily to the aquaria bottom. This was usually followed by dash swimming. This sequence was repeated several times before the fish lost balance, lay flat on the bottom (exertion), tail beat stopped, followed by cessation of opercular movement and then death (non-response to tactile stimuli).

Table B.9.11: Tail and opercular beat frequency (TBF and OBF) and cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

Variable	Time of exposure (hours)				
	12	24	48	72	96
TBF/min.	5.45 ± 2.73^{b}	15.49 ± 4.41^{ab}	7.87 ± 4.41^{ab}	21.12 ± 5.22^{b}	4.49 ± 5.40^{b}
OBF/min.	113.50 ± 7.23^{a}	113.10 ±7.60^{a}	115.23 ±7.23^{a}	10.79 ± 8.99^{a}	65.15 ± 9.15^{b}
Cum. mortality	6.67 ± 6.86^{e}	37.227 ± 31.21^{d}	56.11 ± 36.16^{c}	63.89 ± 32.39^{b}	73.33 ±29.31^{a}

	Concentration of Roundup (mg/l)						
	0.0	4.0	16.0	18.0	20.0	22.0	24.0
TBF/min.	15.73 ±4.41^{a}	5.79 ±4.41^{a}	16.46 ±4.41^{a}	9.00 ±4.63^{a}	7.04 ±5.92^{a}	0.00 ±0.00	0.00 ±0.00
OBF/min.	96.71 ±7.60^{bc}	123.27 ±8.00^{ab}	108.58 ±8.00^{a-c}	100.93 ±8.00^{a-c}	73.42 ±10.19^{c}	124.72 ±16.99^{ab}	136.99 ±16.99^{a}
Cum. Mortality	0.00 ±0.00	22.67 ±12.80^{d}	24.00 ±24.43^{d}	32.67 ±19.81^{c}	50.67 ±37.70^{b}	77.33 ±36.15^{a}	77.33 ±37.89^{a}

Means with the same superscript in the row are not significantly different (p>0.05)

Table B.2.1-3-3: Regression lines of best fit for the prediction of the values of OBF/min., TBF/min. and cumulative mortality of *C. gariepinus* exposed to acute levels Roundup for 96 hours

Dependent Variable	Independent variable	Prediction equation	Curve type	r^2
TBF	Time	$Y=2.8415e^{0.221x}$	Exponential	0.9828
TBF	Concentration	$Y=0.0017x^2 +0.0192x$	Power	0.9442
OBF	Time	Y=23.314Ln(x) +16.325	Logarithmic	0.9186
OBF	Concentration	Y=25.117Ln(x) +19.269	Logarithmic	0.9812
Mortality	Time	Y=0.513x	Linear	0.9922
Mortality	Concentration	$Y=0.0021x^2 + 0.2451x +4.4278$	Polynomial	0.9722

Where x = independent variable, y= dependent variable

Cumulative mortality of exposed fish was very variable relative to the concentration of the herbicide

(Figure 1).

The cumulative mortality differed with the time of exposure ($p < 0.01$), concentration of toxicant ($p < 0.001$) and interactions between exposure duration and herbicide concentration ($p < 0.01$, Figure 1). Exposed fish produced copious amount of mucus on the gill and skin which appeared to be concentration-dependent in exposed fish with minimal amount on the control group. Pigmentation of the skin of the fish was not noticed in any of the exposure concentrations.

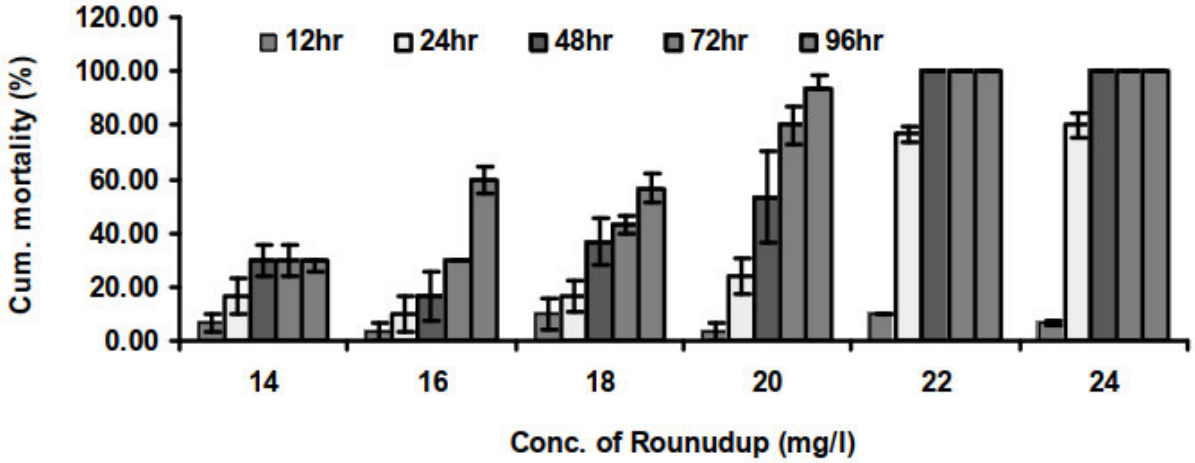


Figure 1: Percentage cumulative mortality of fingerlings of *C. gariepinus* exposed to various concentrations of Roundup for 96 hours

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits of the herbicide concentrations shown below indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of C. gariepinus were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The time it took for half of the exposed fish to die at the various exposure concentrations decreased with time with the highest concentration (24 mg/l) killing half of the exposed fish at about one sixth the time it took for 14 mg/l of the herbicide.

Table B.2.1-4: Lethal concentrations and associated 95% confidence limits of Roundup to *C. gariepinus* fingerling exposure to Roundup for 96 hours

Time (hours)	Lethal Concentration	Safe concentration	Probit model estimation equation
24	LC_{50}- 20.81 (19.58-22.48)	2.08	y= -4.73+0.23x
24	LC_{90}- 26.44 (24.45-31.47)	2.64	
48	LC_{50}-18.50 (16.67-19.40)	1.85	y= -5.18+0.29x
48	LC_{90}-22.54 (20.88-26..09)	2.25	
72	LC_{50}-17.11 (16.30-17.84)	1.71	y= -5.30+0.31x
72	LC_{90}-21.44 (20.59-23.31)	2.14	
96	LC_{50}-15.88 (14.99-16.64)	1.59	y= -5.06+5.06x
96	LC_{90}-19.91 (18.97-21.42)	1.20	

Where y=dependent variable, x= independable variable

Discussion

The threshold concentration causing 100% mortality in this study was 22 mg/l which is lower than that reported for other toxicants tested on any of the clariid species 7 suggesting that it may be more toxic than other tested toxicants. Half of the exposed fish (50%) were killed by 15.88 mg/l of herbicide in 19.69 hours, hence the herbicide can be classified as being slightly toxic. Besides, in the wild where the agro-chemical is indiscriminately used the impact of the exposure stress caused by the herbicide, may be protracted, following the survivors throughout life and may affect various aspects of their lives.

Conclusion

The 24, 48, 72 and 96 hour LC_{50} and associated 95% confidence limits indicated that the range of the values between the 24 hour and 96 hour LC_{50} (4.93 mg/l) as very narrow. Safe concentrations of Roundup to fingerlings of *C. gariepinus* were very low (2.08 mg/l for 24 hour and 1.59 mg/l for 96 hour). The 96 hour LC_{50} of Roundup on the fish was 19.58 mg/l.

Assessment and conclusion

Assessment and conclusion by applicant:

The effects of Roundup containing 360 g/l glyphosate (equivalent to 480g/L isopropylamine salt) were tested in an acute test with *C. gariepinus* fingerlings. The 96 hour-LC_{90} was determined to be 19.91 mg product/L.

There is no analytical verification of test concentrations reported and thus the reliability of the endpoint is questionable. The appearance of mucus accumulation on the skin and gills and skin pigmentation recorded in fish in the holding / stock vessels is a clear indicator of stress. Therefore, the condition of the fish used in the test is questionable. The study was not conducted in accordance with a recognised test guideline and was not performed under conditions of GLP. Furthermore, the purity of the formulation roundup is not clearly given as the specification in the full text contains some typing errors. The study is considered reliable with restrictions.

Assessment and conclusion by RMS:

The conclusion reported above by the applicant is partial. Not only lethal effect but also sublethal, i.e. opercular beat frequency, tail beat frequency, are measured in this study.
The relevance of these sublethal effects for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level (this latter being the specific protection goal). So only results on mortality were considered in deep by RMS. Nevertheless, a link between these abnormal behaviors may exist and may be indicative of mortality and/or potential adverse effect at population level in natural conditions. So, the results for sublethal effects were also reported in the summary and may be considered in future, together with other data available for the active substance.

The present study assessed the acute toxicity (lethal and sublethal) of the glyphosate based formulation Roundup. It is then not known if the high toxicity measured in this study is due to the formulation (and its co-formulants) or a species-specific sensitivity.
The study was conducted with the African catfish *Clarias gariepinus*. RMS considers that the sensitivity of this species can be considered representative of European catfish species.

The applicant notes that there is no analytical verification of test concentrations reported and thus the reliability of the endpoint is questionable. RMS agrees that the absence of analytical verification is a severe drawback of the study. Dose relationship was observed indicating that dosing was somehow adequate, nevertheless uncertainty remains on the actual concentrations.
The applicant notes that mucus accumulation on the skin and gills and skin pigmentation were recorded in fish in the holding / stock vessels. To RMS understanding, "recorded" only means that it was part of the study design (not that it was observed in control). The study author noted that mucus accumulation was concentration-dependant and minimal in the control.

The authors derived what they called "Safe concentration" by multiplying the lethal concentration by a factor 0.1. RMS does not consider these values relevant for risk assessment.

The dissolved oxygen value reported in the study is of 0.01 ± 0.05 mg/l. RMS considers this as a typing error (control fish would have not survived).

The 96 hour LC50 of Roundup on the fish was 15.88 mg/l (equivalent to approximately 5.7 mg glyphosate acid equivalent/L). However, the similarity of the formulations (Roundup vs. MON 52276) is not established.

RMS considers this study being less relevant but supplementary (formulation issue). The data are considered not reliable. RMS cannot discard higher sensitivity of this species (which can be considered representative of European catfish species). However, the similarity of the formulations (Roundup vs. MON 52276) is not established.

Data point:	CA 8.2.2.1, CP 10.2.3
Report author	Lugowska K.
Report year	2018
Report title	The effects of Roundup on gametes and early development of common carp (*Cyprinus carpio L*)
Document Source	Fish physiology and biochemistry (2018), Vol. 44, No. 4, pp. 1109 - 1117
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

To determine the effects of Roundup, a commercial formulation of glyphosate, embryos of common carp (*Cyprinus carpio L*) was exposed to wide range of herbicide concentrations (0.0, 0.1, 0.5, 2.0, 5.0, 10.0, 20.0, and 50.0 mg/L). The obtained results showed different effects of Roundup on common carp gametes. Herbicide reduced swelling of eggs (but the effect was not concentration-related),while sperm showed low sensitivity to Roundup (time of spermatozoa motility was reduced in a significant

way only at 20 mg/l, and at remaining concentrations, only a slight tendency was observed).During the embryonic development, Roundup caused a decrease of common carp embryonic survival (and the effect was concentration-related); however, it had no effect on development rate. During the embryogenesis, three types of embryo body malformation were observed: yolk sac edema, spine curvature, and shortening of body, but their frequencies were not associated with the presence or concentration of herbicide. However, Roundup affected quality of newly hatched larvae of common carp by increasing their mortality. No effect of herbicide on percentage of deformed larvae was observed but larvae hatched in water with Roundup tended to show more complex anomalies compared to those from the control. Obtained data showed that even low concentrations of this herbicide in waters can significantly reduce egg swelling, survival of embryos, and quality of fish larvae.

Materials and Methods

Test organism
The study was done on eggs and sperm and embryos of common carp. The eggs and sperm were obtained from the commercial hatchery of fishing farm "Samoklęski" in Kamionka, Poland. Gametes were stripped manually during artificial spawning from mature females and males. The samples of about 3 ml of eggs and 1 ml of sperm from each female or male, respectively, were placed in Eppendorf test tubes. The eggs from three females and sperm from five (common carp) males were fertilized in about 2 h after stripping by pooling the eggs from all females and sperm from all males and mixing with a small amount of water (the temperature 22 °C) for 3 min.

Media preparation
Fertilized eggs were incubated in de-chlorinated tap water (dissolved oxygen saturation about 80%; hardness 167 mg/dm^3 as $CaCO_3$; pH 7.8, temperature 22 °C) containing different concentrations of Roundup. The Roundup Ultra 170 SL Transorb containing 41% glyphosate acid equivalent, glyphosate concentration 170 g/L (Monsanto Europe SA/NV).

Sperm analysis
Motility of spermatozoa was measured using light microscope (magnification 40 × 12.5), after combining 10 µl of sperm with 10 µl Roundup solution (0.0, 0.1, 0.5, 2.0, 5.0, 10.0, 20.0, or 50.0 mg/L, as glyphosate) on a glass slide in order to activate the spermatozoa. The sperm concentration was not measured, using any specific method. Time of sperm activity was measured from the moment of activation until cessation of spermatozoa movements in three randomly chosen slide fields (five replicates for each concentration).

Egg analysis
For evaluation, the effects of Roundup on egg swelling 25 fertilized eggs were placed in Petri dishes for egg diameter measurement containing Roundup (0.0, 0.1, 0.2, 0.5, 2.0, 5.0, or 10.0 mg/L, as glyphosate). Diameters of whole egg and the yolk were measured 20, 40, 60, and 120 min after fertilization using stereoscopic microscope (magnification 1.6 × 12) to establish exact time of maximum swelling (in fisheries swelling is traditionally measured after 120 min). The percent of swelling was calculated as the following: $S = (c - d) \times 100/d$, where *S*-swelling (as increase in egg diameter), *c*-egg diameter, and *d*-yolk diameter (base line). As a base yolk diameter was used, because before swelling, egg diameter is equal to the yolk diameter (there is no perivitelline space which develops and increases during swelling).

Embryo incubation
Development of common carp embryos took place in 2 L aquaria containing different Roundup concentrations (0.0, 0.1, 0.2, 0.5, 2.0, 5.0, or 10.0 mg/L, as glyphosate). Embryos in aquaria were incubated in additional glass dishes of 8 cm diameter ($n = 4$) in which just after fertilization were place about 45 eggs. All embryos were observed three times a day to evaluate the development. Time of achievement of each development stage was evaluated (when more than 50% of embryos in group reached particular stage), deformed embryos were counted, and cumulative percent of deformations was calculated at the stage of cleavage and organogenesis. Dead embryos (whitish opaque eggs) were counted and removed at five embryonic stages (body formation, metamere formation, development of

eye, and brain germs, before hatching and at hatching) to calculate embryonic survival.

Larvae analysis

Newly hatched larvae were counted and inspected to evaluate their quality. The larvae were divided into three groups: normal (live, motile, without visible abnormalities), deformed (live, moving erroneously, showing body malformations), dead (immobile, opaque, whitish). The percentage of each group among the entire pool of hatched larvae was calculated. The deformed larvae were photographed and classified. The number of different types of deformations in each group was counted as a percent comparing to the total number of deformed larvae in particular group (without statistical analysis).

Embryos and larvae were observed and photographed using a computer MultiScan image analysis system and stereoscopic microscope Nikon SMZ-2 T. For observation of embryos, each glass dish in which development took place was transferred to the microscope table and all embryos were inspected without any handling. Newly hatched larvae were harvested using plastic pipette with wide opening and placed separately on concave glass slide filled with small amount of adequate solution of Roundup.

Analysis of data

Normality of distribution was tested by the Shapiro-Wilk's test and homogeneity of variance using Levene's test. Only one parameter-egg swelling-showed normal distribution, and the results were analyzed by ANOVA, followed by Tukey's post hoc test. For the data that did not meet the assumptions of ANOVA (survival of embryos, percentage of deformed embryos, quality of newly hatched larvae), a non-parametric *U* Mann–Whitney test was performed. The level of significance was set at $p < 0.05$. Data were presented as means ± SD. Results were analyzed using STATISTICA 10 program.

Results

The effects of Roundup on gametes

Eggs of common carp eggs in control group swelled already in 20 min from fertilization (Fig. 1a). Swelling eggs in Roundup groups did not depend on concentration. Comparing to the control result (39%) percent of swelling was significantly lower in groups R0.1, R5, and R10. Among Roundup-incubated groups, the highest swelling was observed in R0.5 and R2 (results with no significant difference to control and between groups). After 120 min (Fig. 1b), only in group R0.1 and R10, egg swelling was significantly lower (the lowest R0.1 28.8%) comparing to the control. Average time of common carp sperm activity at each concentration of herbicide (about 65 s) was lower compared to the control (72.4 s), but no significant differences occurred (except for 20 mg/L-52.6 s) due to high variability (Fig. 2).

Figure 1. a Swelling of common carp eggs (Tukey's post hoc test; different letter superscripts indicate significant differences among experimental groups): after 20 min from fertilization. **b** Swelling of common carp eggs (Tukey's post hoc test; different letter superscripts indicate significant differences among experimental groups): after 120 min from fertilization.

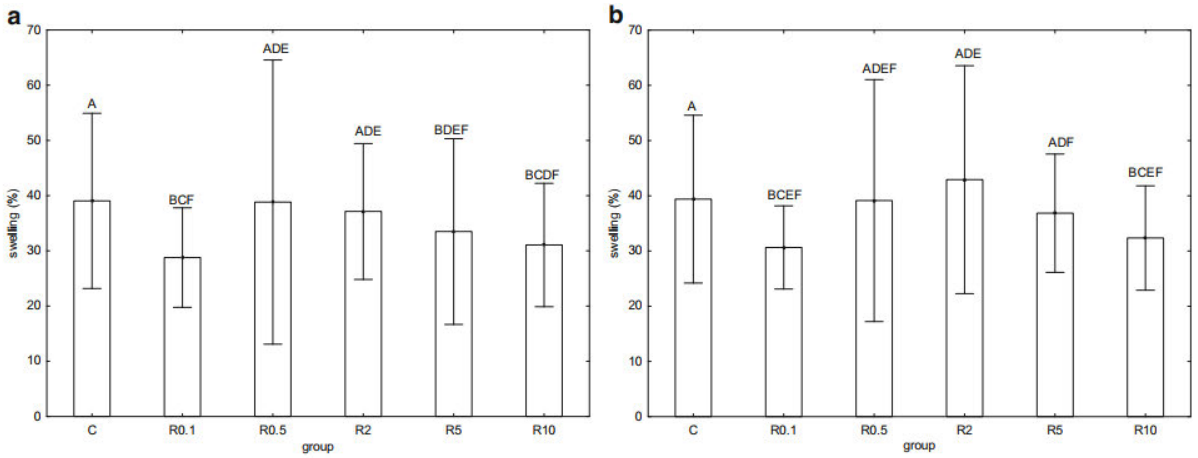
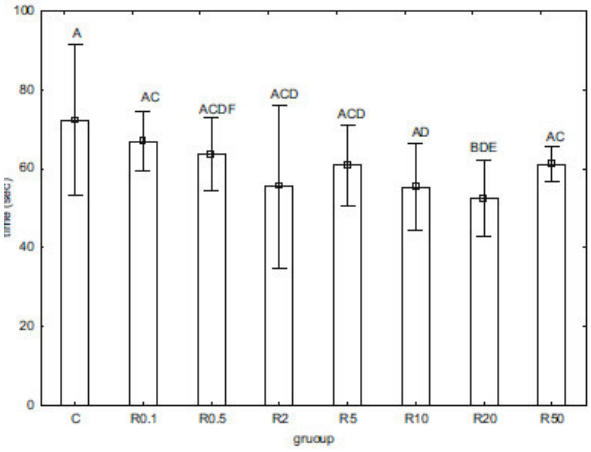


Figure 2. Motility of common carp sperm (U Mann-Whitney test, $p < 0.05$; different letter superscripts indicate significant differences among experimental groups).



The effects of Roundup on embryonic development of common carp

The rate of embryonic development as well as start of hatching (Table 1) was similar in all experimental groups. Roundup caused elongation of hatching and in result a delay of its end (by 1–6 h).

Survival of embryos gradually decreased during the embryonic development and was significantly lower at each stage in groups exposed to Roundup compared to the control (Fig 3). The survival of embryos significantly decreased with increase of herbicide concentration. Hatching percent (as final survival of embryos) in control was 78.5% (the hatching success of common carp under optimum conditions is usually above 70%), whereas at 10 mg/L of Roundup was only 23%. During the embryogenesis, three types of embryo body malformation were observed: yolk sac edema, spine curvature, and shortening of body. These deformations were observed only at 0.1 (1 and 2 in 0.16 ± 0.315% and 3 in 0.16 ± 0.515% of embryos), 0.5 (1 in 0.45 ± 0.614% and 2 in 0.48 ± 0.334% of embryos), and 10.0 mg/l (only 2 in 0.25 ± 0.5% of embryos) of Roundup, but the results did not significantly differ among the groups. The highest percentage of newly hatched normal larvae was obtained in the control group (97%) with only small amount of deformed and dead ones. In all groups exposed to Roundup percentage of normal larvae decreased, while the frequency of dead larvae significantly increased, significant effect of herbicide on percentage of deformed larvae was observed 0.1 and 5 mg/L. In the control, deformed larvae showed only two types of malformations: spine curvatures (about 77%) and deformations of yolk sac. Similar results were observed at 0.1 mg/L of Roundup, while at higher concentration frequency and complexity of deformations increased. At 0.5 mg/L, heart edema occurred, and 2.0, 5.0, and 10.0 mg/L, heart and yolk sac edema were observed together with spine curvature or head deformations.

Table 1. The effects of Roundup on the rate of common carp embryonic development (time in hours post fertilization-hpf).

Stage of embryonic development (hpf)	Concentration of Roundup (mg/l)					
	0.0	0.1	0.5	2.0	5.0	10.0
2 blastomeres	1.3	1.3	1.3	1.6	1.6	1.6
8 blastomeres	2.3	2.3	2.3	2.6	2.6	2.6
Small-cell blastula	3.0	3.0	3.0	3.2	3.2	3.2
Formation of embryo body	14.0	14.0	14.0	14.0	14.0	14.0
Formation of metameres	23.0	23.0	23.0	23.3	23.3	23.3
Eye germs and brain formation	36.0	36.4	36.4	36.4	36.4	36.4
Eye pigmantation	40.0	40.0	40.0	40.0	40.0	40.0
Heart movements	41.0	41.3	41.3	41.0	41.3	41.3
Body pigmentation	54.0	54.2	54.6	54.2	54.2	54.2
Blood coloration	64.0	64.0	64.5	64.0	64.0	64.0
Start of hatching	81.5	82.0	82.2	81.5	81.5	81.0
End of hatching	100.5	106.5	106.5	104.0	104.0	101.5

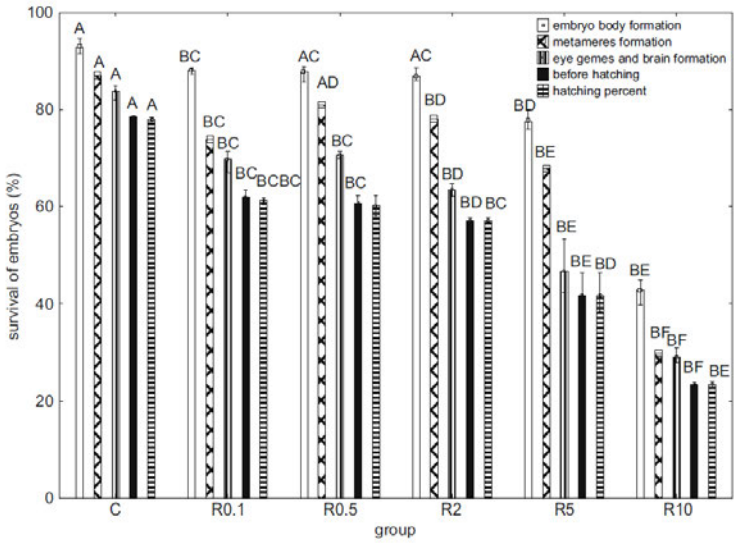


Fig. 3 Survival of common carp embryos (different letter superscripts indicate significant differences among experimental groups, *U* Mann–Whitney test, $p < 0.05$)

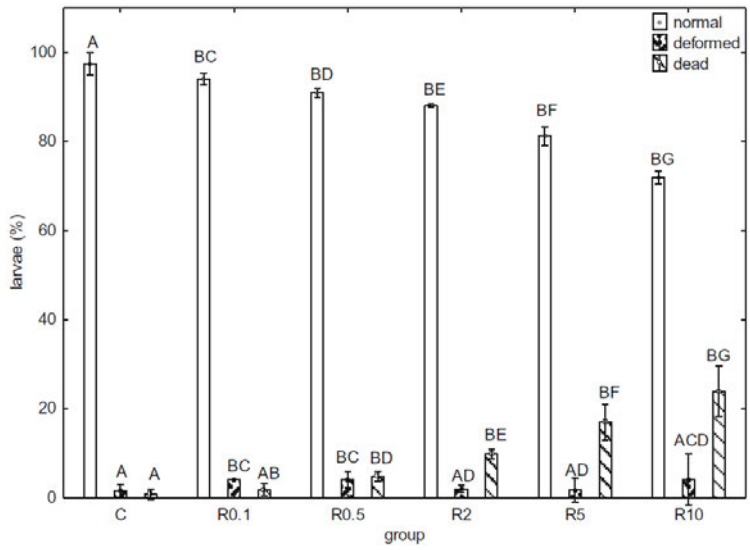


Fig. 4 Quality of newly hatched larvae of common carp (different letter superscripts indicate significant differences among experimental groups, *U* Mann–Whitney test, $p < 0.05$)

Conclusion

Roundup reduced swelling of common carp eggs, but the effect of Roundup was not concentration-related. The results showed low sensitivity of common carp sperm to Roundup that reduced time of spermatozoa motility in significant way only at concentration 20 mg/L and at remaining concentrations, only a slight tendency was observed.

Roundup caused a decrease of common carp embryo survival, and the effect of herbicide increased with increase of its concentration, but development rate in all groups was very similar. As the survival of the embryos decreased, the hatching rate also decreased with the increase of herbicide concentration.

During embryonic development, three types of body malformations occurred: yolk sac edema, spine curvature, and shortening of body but their frequencies were not associated with the presence or concentration of herbicide.

A decrease in quality of newly hatched larvae compared to the control was observed. All groups exposed to Roundup mortality during hatching resulting in emergence of dead larvae significantly increased. Although the herbicide did not significantly affect frequency of all deformed larvae, its effect on frequency of various types of malformations was visible. Larval deformations caused by herbicide exposure were very similar to those induced by any other toxicants and/ or adverse environmental factors. The study

pointed possible effect of Roundup on common carp early development, an important species in central Europe fish farming. Obtained data showed that even low concentrations of this herbicide in waters can significantly reduce egg swelling, survival of embryos, and quality of larvae.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The material and methods part of the study lack some important information. The preparation of test solutions is missing. The time course of the experiment is unclear. Furthermore, there was no analytical verification of test concentrations reported. Suitable exposure throughout the study was not demonstrated and thus the reliability of the study is questionable. The performance / validity of the test cannot be confirmed as there was no positive control included validity criteria were not stated. No regulatory endpoint useful for risk assessment is given. The study is not to a guideline and is not GLP. The tested formulation is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

This study investigates parameters of interest for the risk assessment. Full summary is therefore reported despite the drawbacks of the study.

The effects of Roundup Ultra 170 SL Transorb on embryos of common carp (Cyprinus carpio L) were investigated with wide range of concentrations (0.0, 0.1, 0.5, 2.0, 5.0, 10.0, 20.0, and 50.0 mg/L). Effects of Roundup Ultra 170 SL Transorb on common carp gametes were observed. Herbicide reduced swelling of eggs (but the effect was not concentration-related), while sperm showed low sensitivity to Roundup Ultra 170 SL Transorb (time of spermatozoa motility was reduced in a significant way only at 20 mg a.i./l, and at remaining concentrations, only a slight tendency was observed). RMS notes the absence of clear conceptual link between swelling of eggs, sperm motility and the specific protection goals for fish. It is agreed that it may play a role in the population health, but such link is not immediate in conceptual terms and not quantifiable. The results on these parameters are not relevant for the risk assessment.

During the embryonic development, Roundup caused a decrease of common carp embryonic survival (and the effect was concentration-related); Survival of embryos gradually decreased during the embryonic development and was significantly lower at each stage in groups exposed to Roundup compared to the control.

During the embryogenesis, three types of embryo body malformation were observed: yolk sac edema, spine curvature, and shortening of body, but their frequencies were not associated with the presence or concentration of herbicide.

Roundup affected quality of newly hatched larvae of common carp by increasing their mortality. No effect of herbicide on percentage of deformed larvae was observed but larvae hatched in water with Roundup tended to show more complex anomalies compared to those from the control.

NOECembryonic survival < 0.1 mg glyphosate/L (lowest tested concentration)

This study doesn't follow a specific guideline (no validity criteria available for these parameters but the performance of control and the dose-effect relationship provides some reliability to the results). No analytical verification of test concentrations is reported. Only graphics are available (no biological data presented in the report).
Roundup Ultra 170 SL Transorb was used. RMS notes that co-formulants are not stated.

The study is less relevant but supplementary (difference of formulation tested with representative formulation not clear) and not reliable.

Data point:	CA 8.2.1
Report author	Ayanda O. I. *et al*.
Report year	2015
Report title	Acute toxicity of glyphosate and paraquat to the African catfish (*Clarias gariepinus*, Teugels 1986) using some biochemical indicators
Document Source	Tropical zoology (2015), Vol. 28, No. 4, pp. 152
Guidelines followed in study	OECD guideline No. 203
Deviations from current test guideline	No analytical measurements of test concentrations were taken
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

The impact of acute exposure of *Clarias gariepinus* juveniles to commonly used herbicide glyphosate was evaluated through changes in fish mortality and biochemical indexes. Juveniles of the African catfish were exposed to varying acute concentrations. After 96 h of exposure, the 96 h LC_{50} for glyphosate was found to be 0.530 mg/L. After the exposure period, some enzymes were assayed for in fish liver using a combination of conventional methods and commercially available kits. Comparing with the control, ANOVA analysis revealed that there were significant ($p < 0.05$) increases in the activities of all enzymes assayed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). There were also significant ($p < 0.05$) increases in the activities of the antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and in lipid peroxidation, which was measured by thiobarbituric acid substances (TBARS). The result revealed that glyphosate has the ability to modulate certain fish biochemical parameters and can therefore serve as reliable indicators of toxicity in environmental impact assessment programs.

Materials and methods

Collection and fish maintenance
Juveniles of *C. gariepinus* were purchased from a fish farm in Ota, Ogun State, Nigeria. The *Clarias* species averaging 7.35 ± 2.33 cm standard length and body weight of 3.94 ± 1.51 g were used for the study. The fishes were conveyed in a well-aerated container into the treatment tanks. Stocks of 10 fishes each were held in containers of 25 L capacity and acclimatized for two weeks in dechlorinated water. During this period, the fishes were fed with pelleted diet containing 35% crude protein, twice per day at 4% body weight. The fishes were thought to have adapted to the laboratory conditions when less than 5% death was recorded after 14 days. Feeding was interrupted 24 h before the start of the experiments.

Preparation of test solutions and exposure of fish
Glyphosate was purchased from a commercial outlet in Lagos, Nigeria. Static bioassay was conducted in the laboratory following OECD guideline No. 203, to determine the toxicity of glyphosate to *C. gariepinus*. For each trial, five concentrations, namely 0.36, 0.48, 0.60, 0.72, 0.84 mg/L for glyphosate and a control (0.00 mg/L) were dispensed into 150 L tanks containing dechlorinated water connected to three 25 L tanks. Ten fishes were randomly distributed into each test tank and this was replicated three times. Also, the physico-chemical parameters of the diluting water (temperature, pH, dissolved oxygen, total hardness, total alkalinity and conductivity) during the acute test were measured. The dechlorinated tap water used had the same physical and chemical properties with the one used in acclimatizing the fish. The control solutions were made up of only dechlorinated tap water. All five concentrations of glyphosate were administered to the fish tanks once and the response of the fishes was monitored for 96 h. During this period, dead fishes were removed, counted and recorded.

Tissue sampling
After 96 h, fishes were removed from each trial, sacrificed by cervical decapitation and their livers excised. The liver was washed in ice-cold 1.15% KCL solution, and weighed. They were then homogenized in homogenizing buffer (50 mM Tris-HCl mixed with 1.15 KCL and pH 7.4) using a motor-driven Teflon homogenizer. The resulting homogenate was centrifuged at 12,000 g for 10 min at 4°C. The clear supernatants collected were used for protein estimation and assaying the activity of enzymes.

Biochemical profiling and processing of fish tissues
Commercial kits were purchased for the determination of the levels of liver-damaging enzymes. These include ALT, AST, LDH and ALP. Prior to measurement of enzyme levels, the fish tissue was processed. Glutathione peroxidase (GPx) activity was assayed by the method of Paglia and Valentine (1967) with modifications according to Lawrence and Burke (1978). The result was expressed as mmol/min/mg protein. Catalase (CAT) activity was assayed by the method of Claiborne (1985). Superoxide dismutase (SOD) activity was assayed by the method of Misra and Fridovich (1972). The result of the activities of both enzymes was expressed as unit/mg protein. The assay was based on the ability of SOD to inhibit the autoxidation of epinephrine at alkaline pH. The malondialdehyde (MDA) content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid substances (TBARS) using the method of Buege and Aust (1978). Result expressed as μ mol^{-1} fresh weight.

Statistical analysis
Probit analysis was used to calculate LC_{50} value. The data gathered from the analysis of biochemical parameters were subjected to analysis of variance (ANOVA) using the GenStat 4.2 (2006) statistical analysis software and Duncan multiple range test (DMRT) was used to test for differences between levels of treatment and to separate means respectively, where applicable (Duncan 1955). Test of significance was at 5% level.

Results

Table 1 Physicochemical parameters of diluting water monitored during experiment with glyphosate.

Parameters	Range	Mean ± SE
Temperature (°C)	28.2–30.8	29.52 ± 0.06
pH	6.04–7.14	6.59 ± 0.09
Dissolved Oxygen (mg/l)	3.7–5.7	3.98 ± 0.18
Conductivity (μscm-1)	138–190	160 ± 9.44
Hardness (mgl^{-1} CaCO3)	30–44	36.6 ± 0.78
Alkalinity (ml^{-1})	10–76	40 ± 3.38

Mortality/96-h LC_{50} values
Fish mortality was observed with every concentration of glyphosate. Mortality was observed to increase with increasing concentrations and the highest mortality was recorded in the highest concentrations. The number of deaths, corresponding to each concentration of the herbicide, and the percentage mortality are presented in Table 2. The 96-h LC_{50} value was also calculated based on these calculations and was found to be 0.530 mg/l for glyphosate.

Table 2. Mortality and probit values of *C. gariepinus* exposed to acute concentrations of glyphosate for 96 h.

Conc. (mg/l)	Log $_{10}$ conc.	Total number of fish exposed	Number of dead fish	% mortality	Probit value
0.00	0	30	0	0	0
0.36	− 0.444	30	5	16.67	4.05
0.48	− 0.319	30	10	33.33	4.56
0.60	− 0.222	30	20	66.67	5.44
0.72	− 0.143	30	24	80	5.84
0.84	− 0.076	30	26	86.67	6.13

Biochemical effects

The result of the activities of all the enzymes (ALT, AST, LDH, ALP, CAT, SOD, GPx and lipid peroxidation) assayed for after exposure of juveniles of *C. gariepinus* to acute concentrations of glyphosate are presented in Tables 3 and 4.

Table 3. Activities of liver enzymes after exposure to acute concentrations of glyphosate.

Concentrations (mg/l)	ALT (U/l)	AST (U/l)	LDH (U/l)	ALP (U/l)
0.00	62.318 ± 1.87^{a}	60.918 ± 1.589^{a}	70.575 ± 1.106^{a}	21.888 ± 1.840^{a}
0.36	63.22 ± 3.881^{a}	64.403 ± 1.658^{b}	78.65 ± 3.887^{b}	24.495 ± 2.088^{b}
0.48	68.52 ± 3.421^{b}	70.6 ± 4.055^{c}	86.475 ± 1.008^{c}	26.739 ± 2.349^{c}
0.60	72.108 ± 2.698^{c}	75.523 ± 1.905^{d}	88.513 ± 1.599^{d}	27.647 ± 1.077^{c}
0.72	77.638 ± 3.192^{d}	77.875 ± 1.757^{e}	89.718 ± 2.921^{d}	31.229 ± 2.641^{d}
0.84	80.200 ± 2.207^{e}	82.428 ± 2.877^{f}	91.506 ± 4.844^{e}	33.646 ± 1.857^{e}

Notes: Values with the same superscript in the same column are not significant ($p \geq 0.05$). Mean values ± SE is for four fishes.

Table 4. Activities of antioxidant enzymes and GPx after exposure to acute concentrations of glyphosate.

Concentration (mg/l)	CAT (Unit/mg protein)	SOD (Unit/mg protein)	MDA(μ mol -1)	GPx (mmol/min/mg protein)
0.00	120.877 ± 10.965^{a}	20.170 ± 1.924^{a}	1.258 ± 0.042^{a}	1.971 ± 0.054^{a}
0.36	134.272 ± 8.186^{b}	24.184 ± 2.730^{b}	1.539 ± 0.063^{b}	3.198 ± 0.177^{b}
0.48	136.626 ± 12.055^{c}	27.190 ± 3.683^{c}	1.889 ± 0.076^{c}	3.270 ± 0.092bc
0.60	150.990 ± 11.075^{d}	28.291 ± 2.833^{c}	2.109 ± 0.086^{d}	3.336 ± 0.100^{cd}
0.72	155.555 ± 10.955^{e}	32.401 ± 2.093^{d}	2.168 ± 0.064^{a}	3.383 ± 0.155^{cd}
0.84	158.555 ± 11.218^{f}	35.387 ± 3.578^{e}	2.169 ± 0.053^{d}	3.340 ± 0.174^{d}

Notes: Values with the same superscript in the same column are not significant ($p \geq 0.05$). Mean values ± SE is for four fishes.

Glyphosate caused changes in the activities of ALT, AST, LDH and ALP in fish, suggesting that this herbicide affects the normal functioning of fish. Compared with the control, there were significant increases in the activities of all of these enzymes after 96 h. These increases were also concentration dependent.

After 96 h of exposure to glyphosate, a 77.7% increase in the activities of ALT, 73.90% increase in AST, 77.55% increase in LDH and 65.05% in the activities of ALP, compared to the control was observed.

Changes in the activities of the anti-oxidant enzymes after exposure of fish to glyphosate for 96 h are significant comparing control with treated fishes. In fishes exposed to glyphosate, 76.23% increase was recorded in CAT activities, 57% increase in SOD, 58% increase in MDA and 59.01% increase in GPx activities.

Conclusion

The present study shows that acute concentrations of glyphosate are toxic to fish. After 96 h of exposure, the 96 h LC_{50} for glyphosate was found to be 0.530 mg/L. After the exposure period, some enzymes were assayed for in fish liver using a combination of conventional methods and commercially available kits. Comparing with the control, ANOVA analysis revealed that there were significant ($p < 0.05$) increases in the activities of all enzymes assayed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP). There were also significant ($p < 0.05$) increases in the activities of the antioxidant enzymes: catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and in lipid peroxidation, which was measured by thiobarbituric acid substances (TBARS). The result revealed that glyphosate has the ability to modulate certain fish biochemical parameters and can therefore serve as reliable indicators of toxicity in environmental impact assessment programs.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The test item was not identified, therefore it is not clear what was actually tested and to which compound the effects / results can be assigned. The study is considered unreliable.

Assessment and conclusion by RMS:

The relevance of these biochemical indicators for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level (this latter being the specific protection goal). So only results on mortality were considered by RMS.

The present study shows that acute concentrations of glyphosate are toxic to African catfish *Clarias gariepinus*. After 96 h of exposure, the 96 h LC50 for glyphosate was found to be 0.530 mg/L. LOEL mortality (and biochemical effects) = 0.36 mg/L, with 16.67% mortality.

However there was no test item detailed information available. Exposure is expressed as glyphosate but the form is unknown. Moreover, it cannot be ascertain that commercial formulation (containing surfactants) was not used (test item purchased from a commercial outlet). Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. Because of uncertainty on test item, the study is considered less relevant but supplementary by RMS.

The OECD 203 gave recommendations for the acclimatisation phase indicated that if mortalities are greater than 10% during the first seven days, the batch should be rejected. Here, it was indicated that less than 5% mortalities was obtained after 14 days. There is no indication of the initial sensitivity of the fish to laboratory conditions at 7 days. This may affect the outcome of the test.

No analytical verification is available. Dose effect relationship was observed indicating that dosing was somehow adequate, nevertheless uncertainty remains on the actual concentrations. In absence of clear identification of the product used and its similarity with the current EU representative formulation, RMS considers that this study is of low reliability and cannot be taken into account as a reliable information for the assessment of the active substance glyphosate itself.

Thus, RMS agrees to classify the study as less relevant but supplementary and not reliable enough for risk assessment purpose after detailed assessment of full-text article.

Data point:	CA 8.2
Report author	Cattaneo R. *et al*.
Report year	2011
Report title	Toxicological responses of *Cyprinus carpio* exposed to a commercial formulation containing glyphosate.
Document Source	Bulletin of environmental contamination and toxicology (2011), Vol. 87, No. 6, pp. 597
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant:	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

See RMS analysis in RMS comment box	

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The data on the formulation are not relevant for the glyphosate EU renewal (formulation used is not the representative formulation MON 52276). Roundup tested in this study (480 g/L) contains surfactant other than the EU representative formulation relevant for the glyphosate EU renewal. In this study the formulation tested was used up to 10 mg/L with common carp to look at impact on AChE enzyme and physiological effects. Study is described well but is not conducted to a guideline and the endpoints cannot be extrapolated as enzyme, cellular and molecular level endpoints are not relevant for the EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The effects of commercial glyphosate formulation Roundup on the activity of acetylcholinesterase (AChE) enzyme and oxidative stress were studied in *Cyprinus carpio*.
It is also clearly stated that the surfactant polyethoxylated tallow amine (POEA) was present in the formulation which is known to be more toxic than glyphosate to fish. Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Some effects were observed (even at lowest tested concentration 0.48 mg/L roundup) that can affect the physiological condition of the fish but it is not possible to discriminate between glyphosate and POEA.
Due to presence of POEA, the study is not relevant by RMS for assessment of the current EU representative formulation MON52276. The reliability of the study was not assessed.

Data point:	CA 8.2.1, CP 10.2.1
Report author	Chandrasekera W. U. *et al*.
Report year	2011
Report title	The lethal impacts of Roundup® (glyphosate) on the fingerlings of guppy, *Poecilia reticulata* Peters, 1859.
Document Source	Asian Fisheries Science (2011), Vol. 24, No. 4, pp. 367-378
Guidelines followed in study	None
Deviations from current test guideline	Not applicable, but no analytical measures of the test concentrations were taken
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The material and methods lacks important information. The purity of the formulation is not presented. There is a narrative on water qualities / environmental conditions during the test, but there is no actual data presented to confirm the acceptability of the exposure / test conditions except for a value presented for dissolved oxygen levels. There was no analytical verification of test concentrations reported and therefore the level of exposure cannot be confirmed. The study is considered unreliable.

Further points of clarification:
In the introduction of this paper, the author states that *"..commercial formulations such as Roundup® are more toxic (Langiano and Martinez, 2007) as they contain a surfactant called poly-oxy ethylene amine".* This statement adds uncertainty concerning the supplemental relevance of these findings to the EU renewal of glyphosate, as POEA surfactants are not permitted for use in formulated herbicidal products in the EU. The surfactant system in the formulated product used in this study, was not confirmed by the authors, with only the nominal active ingredient content of the Roundup® being stated. Therefore, the findings of this paper should be treated with a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The lethal effects of commercial glyphosate formulation Roundup were studied on fingerlings of guppies (*Poecilia reticulata*). Further, the behavioural changes of the fingerlings and the histopathological changes of their gills were examined.

In view of the information reported in the article and its year of publication, it is very likely that the surfactant polyethoxylated tallow amine (POEA) was used in the formulation, which is known to be more toxic than glyphosate to fish. Besides, to justify the choice of the test item, the authors report in the introduction that "*although acute toxicity of glyphosate itself is considered to be low, commercial glyphosate formulations such as Roundup® are more toxic as they contain a surfactant called poly-oxy ethylene amine. This surfactant is used to promote penetration of glyphosate through plant cuticle thereby enhancing its efficiency*". Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Effects (lethal and sublethal) were observed but it is not possible to discriminate between glyphosate and POEA.
Due to potential presence of POEA, the study is considered not relevant by RMS for assessment of the current EU representative formulation MON52276. The reliability of the study was not assessed.

Data point:	CA 8.2
Report author	Filizadeh Y. *et al*.
Report year	2011
Report title	Toxicity determination of three sturgeon species exposed to glyphosate.
Document Source	Iranian Journal of Fisheries Sciences (2011), Vol. 10, No. 3, pp. 383-392
Guidelines followed in study	None
Deviations from current test guideline	No analytical measures of the test compound were taken.

GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The present Iranian study was undertaken to evaluate the acute toxicity of glyphosate to three different sturgeon species (*Huso huso*, *Acipenser stellatus*, and *A. persicus*) under laboratory conditions. Fish were exposed to one of ten glyphosate concentrations (10 to 100 mg/L with 10 mg/L intervals), along with a control group. The values of the median lethal concentration (LC_{50}) for each experimental species were estimated using a standard Probit regression analyses after each 6, 12, 24, 48, 96, and 168 hours as exposure times. Results showed that increase in glyphosate exposure times up to 168 hours was simultaneous to decrease of the lethal concentration (LC_{50}). 96-h LC_{50} of glyphosate for *H. huso*, *A. stellatus* and *A. persicus* were 26.4, 23.2 and 27.5 mg/L, respectively. Glyphosate exhibited a slight to moderate toxicity in sturgeon species. However, it may negatively affect the natural population of sturgeons through decreasing of fry mass, smaller size of yolk sac and the initiation of unsafe behaviours.

Materials and methods

Sturgeon fries (weight, 10.2 ± 1.0 g; length, 8.0 ± 0.4 cm) were obtained from International Sturgeon Research Institute (ISRI), Rasht, Iran. Prior to the bioassays, fish were adapted to laboratory conditions for approximately 4 weeks in 400 L tanks with constantly aerated water. They were kept in a static system with constant aeration and natural photoperiod (12:12 h L:D photoperiod). Water quality parameters were daily evaluated throughout the experiment and were as follow: temperature 22 ± 1.5 °C, pH 7.4 ± 0.2 units, dissolved oxygen 6.8 ± 1.3 mg/L, ammonia–N 0.009 ± 0.004 mg/L, nitrite–N 0.02 ± 0.01, and nitrate–N 0.63 mg/L. All water parameters were measured according to APHA (1992). Fish were fed once a day during the acclimation period using commercial pellet food. Feces and pellet remains were removed by suction every day. Acute toxicity tests were carried out in a static way according to the procedure described by Antón et al. (1994). The commercial formulation Roundup® (41% a.s. Monsanto Company, St Louis MO, USA) was used in this study. Experiments were done in 100 L aquariums each containing eight fish, constant aerated water and the same characteristics explained for the adaptation period.

The experiment was composed of ten groups for each species exposed to one of ten glyphosate concentrations (10 to 100 mg/L with 10 mg/L intervals) and a control exposed merely to water (without herbicide addition). All tests were performed in triplicates and no feeding was done during the experiment. The herbicide was added to the aquaria at the beginning of the test. The number of dead fish was recorded for six interval times (6, 12, 24, 48, 96 and 168 h). The value of the mean lethal concentration (LC_{50}) for each experimental species was estimated using Finney's standard probit regression analyses and 95% probability for respective confidence limits (Finney, 1971).

Behavioral changes of experimental fish including swimming speed, anomalous swimming, orientation, and breathing frequency were daily observed and recorded during the toxicity experiment. The findings were examined for conformity to normality with Kolmogorov–Smirnov's test. The SAS statistical program (2008) was used to assess mortality among experiments. The effects of glyphosate concentration for each exposure time were compared to the sturgeon species survival using one–way analyses of variance (ANOVA) with subsequent use of Tukey's HSD test to separate means. The p–value < 0.05 was considered statistically significant.

Results

Glyphosate concentration and exposure times have been recognized as important factors in the survival of sturgeon species. A rise in these factors determines an increase in metabolic rate with negative effects on the survival rate. Many injury appearances were observed in Sturgeon fries in whole concentrations during the study. In case of the control group, no mortality occurred throughout the experiment. Exposure to more than 50 mg/L glyphosate resulted in more than 80% mortality.

The statistical analysis indicated that glyphosate concentrations and exposure times had significant effects on sturgeon mortality. The LC_{50} value obtained for each exposure time of every species was never lower than those obtained for succeeding exposure times. The 6–h LC_{50} and 168–h LC_{50} of glyphosate was the highest and lowest values for each species, respectively (Table 1). The quantitative comparison of LC_{50} exhibited no significant differences between three sturgeon species by increasing exposure duration to 48 h (ANOVA, $p < 0.05$). However, acute toxicity after 48 h exposure time differed significantly between the species (ANOVA, $p < 0.05$). The 168–LC_{50} for experimental species was in a decreasing order of *H. huso* > A. *persicus* > *A. Stellatus* (Table 1).

Results of Finney's probit regression analyses indicated glyphosate has lower acute toxicity to *H. huso* compared to *A. stellatus* and *A. persicus*. However, all sturgeon species exposed to 100 mg/L died within 24 hours from the beginning of exposure. Sturgeon fries exposed to concentrations of 60 to 100 mg/L showed a high increase in mortality.

Normal behavior with no fish mortality was observed in control aquaria during the experiment. After exposure of experimental species to sub lethal concentration of glyphosate (less than 25 mg/L), the fish revealed abnormal behavioral changes such as sluggish swimming, respiratory alterations and excessive mucus secretion. However, fish tended to show more outward signs of toxicity by rising glyphosate concentration. The acutely intoxicated fish exhibited convulsive disorders, loss of equilibrium, color darkening of body surface and finally death compared to the control fish after 196 h.

Table 1. Significant differences between experimental species exposed to glyphosate. Different subscripts within a same row indicate significant differences at $p < 0.05$.

	Huso Huso	*Acipenser stellatus*	*Acipenser persicus*	p–value
6 hours	$74.42 \pm 6.69^{n.s.}$	$69.72 \pm 3.48^{n.s.}$	$77.21 \pm 8.49^{n.s.}$	0.2873
12 hours	$67.19 \pm 5.84^{n.s.}$	$61.15 \pm 11.30^{n.s.}$	$61.34 \pm 14.12^{n.s.}$	0.3324
24 hours	$41.30 \pm 12.39^{n.s.}$	$46.17 \pm 3.69^{n.s.}$	$44.82 \pm 8.51^{n.s.}$	0.2246
48 hours	26.92 ± 3.16^{c}	39.70 ± 4.52^{a}	34.18 ± 6.25^{b}	0.0346
96 hours	19.83 ± 5.35^{b}	24.72 ± 6.73^{a}	26.05 ± 3.20^{a}	0.0117
168 hours	8.31 ± 2.40^{c}	12.98 ± 5.19^{a}	10.59 ± 1.82^{b}	0.0078

n.s. = not significant ($p<0.05$).

Conclusion

This study demonstrated that glyphosate herbicide can be toxic to *H. Huso*, *Acipenser stellatus* and *A. persicus* in concentrations varying from 8.31 to 77.21 mg/L. It means that glyphosate has moderate to low toxicity against these species. The results of the study exhibited that longer exposure times caused greater toxic effects of the bio concentrated chemical and lower glyphosate was needed to reach lethality.

Each sturgeon species exhibited significantly different sensitivity to glyphosate with increasing exposure duration (48, 96 and 168 h) in the order of *H. huso*, *A. persicus*, and *A. stellatus* ($p < 0.05$). The higher sensitivity of *H. huso* may be related to its higher longevity, food behaviors and lifestyles in comparison with *A. stellatus* and *A. persicus*.

The present study confirms that glyphosate is slightly to moderately toxic to fish.

Visual observations demonstrated a series of abnormal behavioral in sturgeon fries, as expected, by increasing exposure time and glyphosate concentration. Nonetheless, normal behaviors of the sturgeon were detected after glyphosate removal, suggesting a lack of herbicide aggregation to the fish tissue.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: LC_{50} generated for sturgeon species. Glyphosate-based herbicide tested. No guideline mentioned but suitable methods described.

Further points for clarification:
The study was not conducted according to a recognized acute fish testing guideline. The product used in the study is not sufficiently identified, but based on the stated '41% a.e. content', this is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). The body weights of the fish used in the study were not reported, therefore fish loading cannot be confirmed and its influence on the observed findings excluded. Chemical analysis was not performed and therefore exposure cannot be confirmed. The impact of a study duration of 168 hours on the fitness of the fish cannot be excluded as having had influence on the observed results, as typically acute toxicity studies with fish are 96 hours in duration.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The study investigates the acute toxicity of glyphosate (in roundup formulation) to three different sturgeon species (*Huso huso, Acipenser stellatus*, and *A. persicus*) under laboratory conditions.
96–h LC50 for H. huso, A. stellatus and A. persicus were 26.4, 23.2 and 27.5 $mg.l^{-1}$, respectively.
These LC50 values are below the other acute endpoints issued from other studies submitted for the EU representative formulation MON 52276. The formulation used in this study (Roundup) is not the EU representative formulation MON52276. In the discussion, the authors indicate that "*the lethal toxicity of commercial toxicant formulation such as Roundup® is more than the glyphosate technical grade substance. The surfactants such as polyethoxylated tallowamine (POEA) used in the Roundup formulation are the principal toxic compound of the glyphosate–based herbicide to aquatic organisms*". It is assumed by RMS that the Roundup formulation used in the test very likely contained POEA). The presence of POEA was also assumed in RAR 2015. The study is then considered not relevant.
Moreover there is no chemical analysis reported in the report to confirm the exposure. Only two values were reported in the discussion part (8.31 to 77.21 mg/L) that seem to indicate rather low recovery (as tested concentrations were between 10-100 mg/L nominal). The authors also noted that many injury appearances were observed in Sturgeon fries in whole concentrations during the study (the reason of that is unknown but this statement questions the condition of the fish in the test). No biological observations is reported, only LC50 values are given. The reliability can not be assessed properly.
The study is considered not relevant and not reliable by RMS.

Data point:	CA 8.2.1
Report author	Isaac A. O. et al.
Report year	2017

Report title	Behavioural and some physiological assessment of glyphosate and paraquat toxicity to juveniles of African catfish, Clarias gariepinus.
Document Source	Pakistan Journal of Zoology (2017), Vol. 49, No. 1, pp. 183-190
Guidelines followed in study	OECD Guideline 203
Deviations from current test guideline	Not specified
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The impact of acute exposure of *Clarias gariepinus* juveniles to glyphosate was evaluated through changes in fish behavior and mortality. Juveniles of the African catfish were exposed to 5 concentrations between 0.36 and 0.84 mg/L of glyphosate during 96 hours of exposure. The test comprised 3 replicates with 10 fishes each. The fish responded, exhibiting different behavioral abnormalities like hyperactivity, abnormal swimming, restlessness, loss of equilibrium and haemorrhage. Observation of opercular ventilation count (OVC), tail fin movement rate (TMR) and air gulping index (AGI) showed a marked difference between control and exposed fishes, indicating that the herbicide negatively impacted on these parameters. These behavioral and morphological anomalies became more pronounced with increasing concentrations of the herbicide. Mortality was also observed to be concentration dependent. After 96 hours of exposure, the 96 h-LC_{50} for glyphosate was found to be 0.530 mg/L.

Materials and Methods

Collection and maintenance of fish
Juveniles of *C. gariepinus* were purchased from a fish farm and those which averaged 7.35±2.33 cm standard length and body weight of 3.94±1.51g were used for the study. Ten fish each were held in 25L tanks and allowed to acclimatize for two weeks in dechlorinated water. During this period, the fishes were fed with pelleted diet containing 35% crude protein twice per day at 4% body weight. The fishes were thought to have adapted to laboratory conditions when less than 5% death was recorded during the period; feeding was discontinued 24 h before the start of the experiment.

Preparation of test solutions and exposure of fish
Glyphosate was purchased from a commercial outlet in Lagos, Nigeria. Acute renewal bioassay was conducted in the laboratory following OECD guidelines No 203 to determine the toxicity of glyphosate to *C. gariepinus*. Five concentrations each, 0.36, 0.48, 0.60, 0.72 and 0.84 mg/L for glyphosate and a control (0.00 mg/L) were dispensed into 150L tanks containing dechlorinated water connected to three 25L tanks. Ten fishes were randomly distributed into each test tank and replicated 3 times. The physicochemical parameters of the diluting water (temperature, pH, dissolved oxygen, total hardness, total alkalinity and conductivity) during the acute test period were measured. The control solutions were made up of only dechlorinated tap water. The five concentrations were administered to the fish-holding units once and the response of the fishes was monitored for 96 h.

Behavioural studies
The fishes were exposed to the different concentrations and observations were made on their behavioural and morphological responses at 12, 24, 48, 72, and 96 h. The control fishes were simultaneously monitored along with the exposed fishes to establish a reference for any behavioural or morphological change. Responses different from the control and occurring in at least 10% of the fish in each test tank were recorded. The behavioural and morphological responses monitored included loss of equilibrium, startle responses, hyperactivity, abnormal swimming, haemorrhage and general restlessness. Startle responses were monitored using the following stimuli of overhead moving visual stimulus, vibration stimulus and tactile stimulus. Air gulping index, opercular ventilation count, tail fin movement and mortality. Air gulping index (AGI) was determined as the number of air gulping activity of fish per tank per minute, divided by the number of fish or surviving fish in the exposed groups. The opercular ventilation count (OVC) and tail fin movement rates (TMR) were determined using a stop watch for two minutes and the average recorded. Fishes were considered dead when the opercular movement ceased and there was no response to gentle probing. This was used as a measure of mortality.
The LC_{50} was determined graphically from a table of probit values.

Results

Effect on fish behavior
The response of the fish juveniles to different behavioural and morphological features after exposure to acute concentrations of glyphosate are presented in Table 1. The control fishes did not show any signs of abnormal behaviours. Acute concentrations were toxic to juveniles of *C. gariepinus*. The behavioural and morphological indexes of toxicity studied, (loss of equilibrium, startle responses, hyperactivity, abnormal swimming, haemorrhage and general restlessness) were all positive to varying degrees. The behavioural abnormalities of the juvenile fishes increased with increasing concentrations. The different concentrations of glyphosate and the durations of exposure also had significant effects on the rate of opercular ventilation of *C. gariepinus*. The highest OVC was observed in the first period of observation i.e. 12 h after exposure. The OVC reduced again by the 24th h, a period which represents the lowest OVC. The OVC increased again by the 48th h and peaked at the 96th h.

Table 1: Behavioural abnormalities of juveniles of *C. gariepinus* after exposure to acute conc. of glyphosate.

Behavioural anomalies	**Concentrations (mg/l)**					
	0.00	**0.36**	**0.48**	**0.60**	**0.72**	**0.84**
Glyphosate						
Loss of equilibrium	-	+	+	+	++	++
Startle responses	-	+	+	+	++	+++
Hyperactivity	-	+	+	++	++	+++
Abnormal swimming	-	+	+	+	+++	+++
Haemorrhage	-	+	+	++	+++	+++
Restlessness	-	+	++	++	+++	+++

–, None; +, Weak; ++, Moderate; +++, Strong.

Table 2: Effect of acute conc. of glyphosate opercular ventilation count of *C. gariepinus* for 12-96 h.

Conc (Mg/L)	Time (h) and OVC				
	12	24	48	72	96
Glyphosate					
0.00	48.00 ± 1.00^{Aa}	50.00 ± 1.00^{Aa}	49.00 ± 1.00^{Aa}	50.00 ± 0.58^{Aa}	50.00 ± 1.00^{Aa}
0.36	46.00 ± 1.15^{Aa}	52.00 ± 1.15^{Ab}	50.00 ± 1.06^{Ab}	54.00 ± 1.15^{Ab}	58.00 ± 0.58^{Ac}
0.48	47.00 ± 1.15^{Aa}	40.00 ± 0.58^{Bb}	48.00 ± 1.03^{Aac}	50.00 ± 1.15^{Bac}	51.00 ± 1.15^{Bac}
0.60	60.00 ± 0.58^{Ba}	35.00 ± 0.58^{Cb}	47.00 ± 1.00^{Ac}	48.00 ± 1.15^{Bc}	50.00 ± 0.58^{Bc}
0.72	61.33 ± 1.15^{Ba}	38.00 ± 0.58^{Cb}	44.00 ± 1.00^{Bc}	45.00 ± 0.58^{Cc}	50.00 ± 0.58^{Bd}
0.84	62.00 ± 1.15^{Ba}	38.00 ± 0.58^{Cb}	45.00 ± 1.02^{Bc}	47.00 ± 1.15^{Bc}	49.00 ± 1.15^{Bc}

Means with the same capital letter superscript along same column and small letter superscript on the same row are not significantly different ($p\geq0.05$); (Mean values ± SE) n =3; OVC, opercular ventilation count.

TMRs were significantly affected by acute concentrations of glyphosate. Interplay between concentration and time revealed that the TMR was highest in the first 12 h of exposure to glyphosate. After this period, TMR reduced by the 24 h and increased again till the 96 h. Comparing with the control, the TMRs in exposed fish were generally lower except during the first 12 h. Interaction between concentration and time showed that all the concentrations had the lowest TMR in the 24 h of exposure. Furthermore, the first 12 h showed the highest TMR, thereafter, it dropped significantly in the 24 h and then increased from the 48 h.

Table 3: Effect of acute conc. of glyphosate on tail fin movement rate of *C. gariepinus* over a period of 12-96 h.

Conc (Mg/L)	Time (h) and TMR				
	12	24	48	72	96
Glyphosate					
0.00	102.00 ± 1.00^{Aa}	100.00 ± 0.58^{Aa}	103.00 ± 1.00^{Aa}	102.00 ± 1.00^{Aa}	103.00 ± 0.58^{Aa}
0.36	130.00 ± 0.58^{Ba}	66.00 ± 1.15^{Bb}	75.00 ± 1.15^{Bb}	77.00 ± 1.15^{Bb}	92.00 ± 0.58^{Bc}
0.48	98.00 ± 0.58^{Ca}	56.00 ± 1.15^{Cb}	78.00 ± 1.15^{Bb}	79.00 ± 0.58^{Bb}	90.00 ± 0.58^{Bc}
0.60	128.00 ± 1.15^{Ba}	71.33 ± 1.15^{Db}	85.00 ± 1.15^{Cb}	87.00 ± 0.58^{Cb}	89.00 ± 1.15^{Bb}
0.72	131.00 ± 0.58^{Ba}	73.67 ± 0.58^{Db}	86.00 ± 1.15^{Cc}	81.00 ± 1.15^{Dd}	88.00 ± 0.58^{Bce}
0.84	127.00 ± 1.15^{Ba}	82.00 ± 1.15^{Eb}	88.00 ± 0.58^{Cc}	90.00 ± 0.58^{Cc}	92.00 ± 1.15^{Bc}

Means with the same capital letter superscript along same column and small letter superscript on the same row are not significantly different ($p\geq0.05$); (Mean values ± SE) n =3; TMR, tail fin movemant rate.

The interaction between duration and concentration effects is presented in Table 4. Acute concentrations of glyphosate showed significant effects on the AGI of *C. gariepinus*. The control fish showed lower AGI when compared with the exposed fish. The highest concentration also showed the highest AGI between the 12th and 48th. This difference is statistically significant. There was no definite pattern in the 72nd and 96th h. The air gulping index in exposed fish was highest within the 12th h and 24th h of exposure.

Table 4: Effect of acute conc. of glyphosate on air gulping index of *C. gariepinus* over a period of 12-96 h.

Conc (Mg/L)	Time (h) and AGI				
	12	24	48	72	96
Glyphosate					
0.00	0.30 ± 0.05^{Aa}	0.40 ± 0.05^{Aa}	0.30 ± 0.06^{Aa}	0.40 ± 0.05^{Aa}	0.40 ± 0.05^{Aa}
0.36	0.50 ± 0.05^{Ba}	0.50 ± 0.05^{Ba}	0.50 ± 0.05^{Aa}	0.30 ± 0.05^{Ab}	0.30 ± 0.06^{Bb}
0.48	0.70 ± 0.05^{Ba}	0.80 ± 0.12^{Cb}	0.80 ± 0.06^{Bb}	0.90 ± 0.05^{Bc}	0.90 ± 0.12^{Bc}
0.60	0.90 ± 0.06^{Ca}	0.70 ± 0.05^{Cb}	0.70 ± 0.05^{Bb}	0.50 ± 0.06^{Cc}	0.50 ± 0.05^{Ac}
0.72	0.80 ± 0.05^{Ca}	0.90 ± 0.05^{Ca}	0.70 ± 0.12^{Bb}	0.30 ± 0.05^{Ac}	0.40 ± 0.05^{Ac}
0.84	1.20 ± 0.05^{Da}	1.20 ± 0.06^{Da}	0.80 ± 0.12^{Bb}	0.50 ± 0.05^{Cc}	0.70 ± 0.05^{Cb}

Means with the same capital letter superscript along same column and small letter superscript on the same row are not significantly different ($p\geq0.05$); (Mean values ± SE) n =3; AGI, air gulping index.

96-h LC_{50} values

Mortality and probit values increased with increasing concentrations with the highest mortality recorded in the highest concentrations. The 96 hour-LC_{50} value was calculated based on these values and was found to be 0.530 mg/L for glyphosate.

Table 5: Mortality values of *C. gariepinus* exposed to acute conc. of glyphosate for 96 h.

Conc. (mg/L)	Log_{10} conc.	Total No. of fish exposed	No. of dead fish	% mortality	Probit value
Glyphosate					
0.00	0	30	0	0	0
0.36	-0.444	30	5	16.67	4.05
0.48	-0.319	30	10	33.33	4.56
0.60	-0.222	30	20	66.67	5.44
0.72	-0.143	30	24	80	5.84
0.84	-0.076	30	26	86.67	6.13

Mortality was observed to be concentration and time dependent in this study. The mortality pattern was between 17% and 87% for glyphosate.

Conclusion

After 96 hours of exposure of African catfish (*Clarias gariepinus*) juveniles to glyphosate, the 96 h-LC_{50} was found to be 0.530 mg/L of glyphosate.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Although the study itself is not directly relatable to an EU level ecotoxicological risk assessment, the study was considered as supplementary only (EFSA GD Point 5.4.1 - relevance category B) as sub-lethal effects on fish behaviour following exposure to glyphosate were described.

Further points for clarification:
A relevant guideline was followed (OECD 203). However, the fish loading was > 1 g fish/L. No water quality data were reported and there was no chemical analysis performed to confirm exposure, so despite being considered supplemental, results should be considered with caution. In addition, it is not clear what was actually tested (active substance or glyphosate formulation; the study is lacking information on purity / composition of the tested item).

Assessment and conclusion by RMS:

The relevance of the effects on behaviour (hyperactivity, abnormal swimming, restlessness, loss of equilibrium) for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level (this latter being the specific protection goal). So only results on mortality were considered by RMS (even if other sublethal parameters are also likely to affect populations, they are not relatable to the risk assessment scheme).
The present study shows that acute concentrations of glyphosate are toxic to African catfish *Clarias gariepinus*. After 96 h of exposure, the 96 h LC50 for glyphosate was found to be 0.530 mg/L. RMS notes that mortality data are identical to those presented in an other study conducted years earlier (Ayanda O. I. et al., 2015, Acute toxicity of glyphosate and paraquat to the African catfish (*Clarias gariepinus*, Teugels 1986) using some biochemical indicators). This study was also assessed by RMS and again, no test substance information was available.
Exposure is expressed as glyphosate but it cannot be ascertain that commercial formulation (containing surfactants) was not used. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. No analytical verification is available. RMS considers that the absence of analytical verification is a severe drawback of the study, particularly when considering the uncertainty regarding the test item used. Dose effect relationship was observed indicating that dosing was somehow adequate, nevertheless uncertainty remains on the actual concentrations.Therefore given the uncertainties on the test item together with the absence of analytics RMS considers that the reliability of the results is very limited. Thus, it cannot be taken into account as critical information or the assessment of the active substance glyphosate itself.

This study is considered less relevant but supplementary (due to the uncertainty on the test item) and not reliable for risk assessment.

Data point:	CA 8.2.1
Report author	Jofre D. M. et al.
Report year	2013
Report title	Fish Toxicity of Commercial Herbicides Formulated With Glyphosate
Document Source	Journal of Environmental & Analytical Toxicology. Vol. 4, no. 1, pp. 1
Guidelines followed in study	None

Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Acute toxicity of two commercial glyphosate-based herbicide formulations (Glacoxan® and Estrella®) was examined. For this purpose ten fish each of the two fish species *Danio rerio* and *Poecilia reticulata* were exposed to a solution of the tested compound starting from a maximum value of 100 µL/L. The mortality rate was documented at 96 hours.
The results indicate that Glacoxan® and Estrella® present acute toxic effects toward *P. reticulata*, since the lowest concentrations that produce 100% of mortality and the maximum concentration that does not produce mortality are of 100 µL/L and 50 µL/L respectively. Estrella® formulation shows a higher acute toxicity toward *D. rerio* since the lowest concentration that produces 100% mortality was 50 µL/L.

Materials and methods

Formulations and solutions
Two commercial glyphosate formulations, present in the local market, were selected. Solution A (Glacoxan®) refers to an herbicide used in gardening; whereas solution B (Estrella®) is an herbicide used in extensive farming practices. Both products were purchased in agrochemical shops in the province of San Luis, Argentina.

Table 1: Characteristics of tested commercial formulations of glyphosate

Property	Herbicide A	Herbicide B
Gliphosate concentration	48 %	48 %
Active compound	Salt isopropilamine	Salt isopropilamine
Excipients	non informed	non informed
pH	5.5	6
Color	Amber	Amber
Transparency	Transparent	Transparent

Acute toxicity assays
Organisms and solutions were placed in a 1 L capacity container. The water level was maintained during the measurements. The essay begins with an initial exposure to potentially toxic agents and continues for 96 hours. Every 24 hour the number of dead specimens in each container were counted and then removed. The mortality rate was documented at 96 h. The toxic effect was evaluated toward two fish species, *D. rerio* and *P. reticulate*. Specimens were bred in the laboratory reaching a maximum size of 1 cm and 15 to 20 days old. Ten fish of each species were exposed to a solution of the tested compound starting from a maximum value of 100 µL/L. The lowest concentration of commercial formulation that produces 100% mortality (LC-100%M) and the maximum concentration that does not produce mortality (MC-0%M) were determined.

Statistical analysis
The chi-square method was used in order to compare the mortality rates depicted in the acute toxicity

tables, meanwhile the nonparametric Student test Mann-Whitney was used for comparisons between the LC-100%M and MC-0%M.

Results

Acute toxicity

For the two fish species, the lowest concentration of the commercial glyphosate formulation A that produce 100% of mortality (LC-100%M) was 100 µL/L and that 50 µL/L was the maximum concentration that does not produce mortality (MC-0%M). Values of 50 µL/L for the lowest concentration of the commercial glyphosate formulation producing 100% mortality (LC-100%M) and 25 µL/L for the maximum concentration that produce no mortality (MC-0%M) were observed when the formulation B was tested toward *D. rerio* specimens.

Table 2: Acute toxicity tests results (A = Glacoxan®, B = Estrella®)

Evaluation of herbicides A and B on *Danio rerio*					
Sample	**Solution conc.**	**Percent of mortality**			
		24 hs.	**48 hs.**	**72 hs.**	**96 hs.**
A-100	100 µL/L	100	-	-	100
A-50	50 µL/L	0	0	0	0
B-100	100 µL/L	100	-	-	100
B-50	50 µL/L	100	-	-	100
B-25	25 µL/L	0	0	0	0
Control	Control	0	0	0	0
Evaluation of herbicides A and B on *Poecilia reticulata*					
Sample	**Solution conc.**	**Percent of mortality**			
		24 hs.	**48 hs.**	**72 hs.**	**96 hs.**
A-100	100 µL/L	100	-	-	100
A-50	50 µL/L	0	0	0	0
B-100	100 µL/L	0	25	100	100
B-50	50 µL/L	0	0	0	0
Control	Control	0	0	0	0

Statistical analysis

The acute toxicity difference is significant when compared the concentrations of 100 µL/L and 50 µL/L obtained for the two solutions tested with a $p \geq 0.0001$. It is also significant ($p \geq 0.0022$) the difference observed in the acute toxicity for both species *D. rerio* and *P. reticulate* (LC-0%M= 25 µL/L and 50 µL/L respectively).

Conclusion

Both tested herbicides, Glacoxan® and Estrella®, presented acute toxic effects toward *P. reticulata*, since the lowest concentrations that produce 100% of mortality and the maximum concentration that does not produce mortality are of 100 µL/L and 50 µL/L respectively. Estrella® formulation shows a higher acute toxicity toward *D. rerio* since the lowest concentration that produces 100% mortality was 50 µL/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The test design and the achieved endpoints are not used in the EU ecotoxicological regulatory risk assessment.

Further point of clarification:
As the study was conducted in Argentina, it is highly likely possible that the glyphosate-based herbicides were purchased from the local market and that the tested formulations are not the representative formulations for the glyphosate EU renewal (the representative formulation is MON 52276). As co-formulants of the formulations tested were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The acute and chronic toxicity of two commercial formulations (Glacoxan® and Estrella®) was investigated. The acute toxicity was tested toward two fish species *Danio rerio* and *Poecilia reticulata* by evaluating the mortality. The chronic toxicity was assessed in D. rerio by measuring the biochemical parameters glutamic pyruvic transaminase (AST) and glutamic oxaloacetic transaminase (ASL).
The relevance of the biochemical parameters for the risk assessment cannot be established by RMS as no quantitative link can be made between these parameters and the potential adverse effect at population level (this latter being the specific protection goal). So only results on mortality were considered by RMS.

In the present study, for the two fish species, Glacoxan® produced 100% of mortality at 100 µL/L and 0% mortality at 50 µL/L (equivalent to 50 mg/L). No intermediate concentration was tested so no robust endpoint can be derived.

Estrella® produced 100% of mortality at 50 µL/L and 0% mortality at 25 µL/L (equivalent to 25 mg/L) on Danio rerio. No intermediate concentration was tested so no robust endpoint can be derived. It produced 100% of mortality at 100 µL/L and 0% mortality at 50 µL/L (equivalent to 50 mg/L) on Poecilia reticulata. Again ,no intermediate concentration was tested so no robust endpoint can be derived.

The nature of surfactant present in the formulations tested is unknown. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. This affects the relevance of the study.
No analytical verification is available. No robust endpoints (LC50) could be derived from the study (as explained above). Thus, RMS considers that this study is not reliable and cannot be taken into account for the assessment of the active substance glyphosate itself.

The study is considered less relevant (different formulation tested) but not reliable.

Data point:	CA 8.2.2

Report author	Panetto O. S. et al.
Report year	2019
Report title	The effects of Roundup® in embryo development and energy metabolism of the zebrafish (*Danio rerio*)
Document Source	Comparative biochemistry and physiology (2019), Vol. 222, pp. 74
Guidelines followed in study	OECD guideline 236
Deviations from current test guideline	Temperature outside the validity criteria limits, being maintained at 28 ±1 ºC for the study duration. Dissolved oxygen level in terms of percentage saturation not reported. Only one other validity criteria mentioned, with respect to control survival, with 2 % mortality achieved in the controls. Fertilization rate success and hatching rate at 96 hours not reported.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The acute 96 hour-LC_{50} for zebrafish embryo after exposure to Roundup was determined to be 58.3 mg/L. Seven test concentrations between 3.5 and 350 mg/L were used with 4 replicates and 20 embryos each. It was stated that the test was performed based on OECD guideline 236. This study type has six validity criteria for the control group, including fertilization rate success (required ≥70% in batch tested), hatching rate at 96 hours (required ≥80%) and overall survival (required ≥90%). There is also a validity criteria requirement for the results of a positive control group, using 3, 4-dichloroaniline, to achieve a minimum of 30% mortality at 96 hours. There are also two water quality criteria relating to water temperature (required 26 ±1 ºC at any time during the test) and for dissolved oxygen at 96 hours to be > 80% of the saturation. Whilst dissolved oxygen levels at 6 mg O_2/L were achieved in the test, the temperature was outside of the validity criteria limits, being maintained at 28 ±1 ºC for the study duration. Therefore the dissolved oxygen level cannot be confirmed as reporting of dissolved oxygen in terms of mg O_2/L requires information on atmospheric pressure and temperature to resolve actual dissolved oxygen in terms of percentage saturation. A slight increase in temperature by a degree Celcius is not overly concerning, however, it is difficult to conclude on the reliability of the study as only one other validity criteria is mentioned, with respect to control survival, with 2% mortality achieved in the controls. There is no information presented on the fertilization rate of the batch of eggs used, nor is there hatching rates presented for the controls or the treatment groups. In addition, the performance of the test system cannot be confirmed as the results of a positive control group were not included. In addition, there are no biological data for the treatment groups presented other than in figures, so the data in the figures cannot be confirmed. Furthermore, claims that the achieved LC_{50} of 58.3 mg/L is 15,000 times lower than that used in agriculture is not supported by corresponding surface water monitoring data. A final point is that the test concentrations in the test system were not analytically verified and therefore, exposure concentrations cannot be confirmed. The study is considered unreliable.

Assessment and conclusion by RMS:

The study authors indicates that Roundup Original®, was used in this study, which presents in addition to glyphosate, surfactants substances that increase the effect power of the herbicide (reference is made to Sánchez et al., 2017).

Sanchez et al 2017 states that « It is known that the surfactant MON 0818, containing POEA, integrates the Roundup Original formulation. The MON 0818 (a code of Monsanto for designation for preparation of POEA) is a mixture of polyethoxylated long-chain alkylamines synthesized from animal-derived fatty acids and is added to facilitate glyphosate penetration into the plants."

This indicates the presence of POEA in the formulation tested.

POEA is not authorized in plant protection products containing glyphosate (European Commission, August 2016).
Due to presence of POEA, the study is considered not relevant by RMS.

Data point:	CA 8.2.1
Report author	Velasques, R. R. et al.
Report year	2016
Report title	Roundup® in Zebrafish: Effects on Oxidative Status and Gene Expression.
Document Source	Zebrafish (2016), Vol. 13, No. 5, pp. 432-441
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The objective of the present study was to determine the effects of Roundup on oxidative status in adult *Danio rerio* liver and gills. Reactive oxygen species (ROS) and antioxidant capacity (ACAP) were measured in fish after exposure to Roundup (5 and 10 mg/L) for 24, 48, 72, and 96 h. Furthermore, gene expression related to antioxidant response was evaluated after 24 and 96 h. In gills, an increase in ACAP was observed after 96 h in the highest concentration. In the liver, a reduction in ROS and ACAP was observed after 24 h, whereas an increase in ACAP was observed after 48 h in the highest concentration. Exposure to the lowest concentration caused a reduction in ROS after 72 and 96 h. Regarding gene expression, a reduction in superoxide dismutase 2 (*sod2*) and glutathione S-transferase (*gstp*) was observed. An increase in uncoupling protein 1 (ucp1) expression was observed in gills of animals exposed to the highest concentration after 24 h. Glutathione peroxidase (*gpx*) gene expression was reduced in the gills of animals exposed to the lowest concentration; however, it was induced in liver tissue after 96 h of exposure to the highest concentration.

Materials and Methods

Fish maintenance and exposure
The animals were obtained commercially from local suppliers and acclimated in aquaria at the Instituto de Ciencias Biologicas from Universidade Federal do Rio Grande-FURG (Institute of Biological Sciences, Federal University of Rio Grande) for a minimum of 15 days. Animals were maintained in 100 L tanks (density of one animal/L) with dechlorinated tap water under constant aeration. Water conditions were maintained at 28 °C, pH 7.0 with a photoperiod of 14L:10D. Nitrite levels were monitored with a commercial kit and maintained at 0 ppm. The animals were fed *ad libitum* every day with commercial food (Tetra-Colorbits). After the acclimation period, juvenile fishes from both genders (234 specimens) were randomly divided into three experimental groups: control group (without Roundup), a group exposed to 5 mg/L of Roundup, and another group exposed to 10 mg/L of Roundup. Experiments were conducted in glass aquaria (6 L, density = one animal/L), under the same conditions as the acclimation period. The animals were exposed for 24, 48, 72, and 96 h in a static system. No mortality was observed during the experimental period. On the final day of the experimental period, animals were euthanized via immersion in metanosulfonated tricaine (500 µg/L; Sigma) and their gills and liver were dissected for subsequent analyses.

ROS quantification
The fluorescent compound dichlorofluorescein diacetate (H_2DCF-DA; Sigma) was employed for ROS quantification. Freshly dissected gills and the liver (pool = 2 animals/n = 6 pools per experimental group) were homogenized in 200 µL of cold homogenization buffer (100mM Tris-HCl, 2mM EDTA, and 5mM $MgCl_2$*$6H_2O$; pH = 7.75). The homogenate was then centrifuged (2000 g for 20 min at 4 °C). The pellet was discarded, and the supernatant was taken for protein measurement with a commercial kit via biuret assay (Doles Reagents LTDA). To standardize protein content between samples, the supernatant was diluted with homogenization buffer to 2 mg/L of protein. After this, 10 µL of the diluted supernatant was added to a microplate with 127.5 µL of reaction buffer (30 mM HEPES, 200 mM KCl, and 1 mM $MgCl_2$; pH= 7.2) and 10 µL of the H_2DCF-DA solution (16 µM). Fluorescence intensity was monitored at 5-min intervals over a 60 min period (25 °C) via a fluorometer (Victor 2; Perkin Elmer) with excitation and emission wavelengths of 485 and 520 nm, respectively. The area between the curve of fluorescence/time was integrated, and the total area was determined for comparison. The results were expressed as percent fluorescence area x·minute (%FA·x min) and compared with the control group (100%).

Antioxidant capacity against peroxyradicals
The diluted supernatants employed for ROS analysis were conserved at -80 °C for further antioxidant capacity (ACAP) determination. The ABAP (2,2-azobis2 methylpropionamidedihydrochloride; 4 mM; Sigma-Aldrich) were used as a peroxyl radicals generator. The ACAP of samples were measured with H_2DCF-DA in the presence and absence of the ABAP. Fluorescence intensity was monitored over a 35 min period at 37 °C via a fluorometer (Victor 2; Perkin Elmer) with excitation and emission wavelengths of 485 and 520 nm, respectively. The difference in fluorescence area with and without ABAP was considered the ACAP. The data were expressed as 1/% relative area compared with the control group, where an increase in ACAP values indicated an increase in the ACAP.

Gene expression analysis
Total RNA from liver and gill samples (pool = 3 animals/ n = 5 pools per experimental group) were extracted with TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. Total RNA were taken for spectrophotometrical analysis (260/280 nm) and electrophoresis gel analysis (agarose 1%) for quality and quantity determination. RNA samples were then standardized by concentration. The complementary DNA (cDNA) was prepared with the High-Capacity cDNA Reverse- Transcription Kit (Applied Biosystems). The cDNAs were diluted and taken for real time PCR following the manufacturer's protocol from the GoTaq qPCR Master Mix (Promega) and the ABI Prism 7300 Sequence Detection System (Applied Biosystems). The specific primers (Table 1) for zebrafish *D. rerio* genes were designed in the Primer 3 at the NCBI website (www.ncbi.nlm.nih.gov/). The efficiency of primers was tested, and only those primers with a reaction efficiency higher than 95% were used. The

analyzed genes were: *nrf2* (Nuclear factor [erythroid-derived 2]-like 2), *cat* (Catalase-hydrogen peroxide degradation), *sod1* (Cu-, Zn-Superoxide dismutase-superoxide anion dismutation), *sod2* (Mn-Superoxide dismutase superoxide anion dismutation), *gclc* (Glutamate cysteine ligase catalytic subunit-glutathione synthesis), *gpx* (Glutathione peroxidase selenium dependent-hydrogen peroxide degradation), *gsta* (Glutathione S-transferase isoform alpha-conjugation with glutathione), *gstr* (Glutathione S-transferase isoformrho-conjugation with glutathione), *gstp* (Glutathione S-transferase isoform pi-conjugation with glutathione), *ucp1* (Uncoupling protein isoform1-mitochondrial proton leak), *ucp2* (Uncoupling protein isoform 2-mitochondrial proton leak), and *ucp3* (Uncoupling protein isoform 3-mitochondrial proton leak). The *tbp* (TATA box binding protein) and *ef1a* (elongation factor 1 alpha) were employed as housekeeping genes. The relative quantification of gene expression was done by the $2^{-\Delta CT}$ method. The results were expressed as fold induction related to the control group (1 standard error).

Statistical analysis
Data were initially assessed to validate analysis-of-variance (ANOVA) assumptions. ANOVAs (with a significance level of 5%) were conducted on data considered normal and homoscedastic to compare groups in the same experimental time. Significant differences were further analyzed via Newman-Keuls *post hoc* test (with a significance level of 5%).

Results

ROS und ACAP

No significant differences were observed in ROS concentration in gills at any experimental time, although a trend of reduction was observed in the first 24 h ($p = 0.058$). ACAP significantly increased (80%) in animals exposed for 96 h to the highest concentration (10 mg/L) compared with the control group ($p < 0.05$). A trend of ACAP induction was also observed in animals exposed to the same concentration at 72 h ($p = 0.0509$).

ROS concentration in the liver significantly reduced in animals exposed to the highest concentration. No significant differences were observed after the 48-h exposure period, although a trend of ROS reduction was observed in both concentrations ($p = 0.065$). After 72 and 96 h, ROS concentration of animals exposed to the highest concentration returned to control level values. However, animals exposed to the lowest concentration presented reduced levels in both experimental times compared with both the control group and animals exposed to the highest concentration ($p < 0.05$). A significant reduction in ACAP levels was observed in animals exposed to the highest concentration of Roundup after 24 h ($p < 0.05$). On the other hand, in a later experimental period, ACAP levels were 1.38 times higher in animals exposed to the same Roundup concentration ($p < 0.05$).

Gene expression
The gene expressions profiles were evaluated in both gills and the liver after 24 and 96 h, respectively, of Roundup exposure. No significant alteration was observed in gene expression of *nrf2*, *cat*, *sod1*, and *gclc* in gills after Roundup exposure for 24 and 96 h ($p > 0.05$). However, after 24 h, the *sod2* gene expression in gill tissue presented a reduction by a factor of 0.59 compared with the control group ($p < 0.05$). After 96 h of exposure, *gpx1a* also presented significant differences in its expression ($p < 0.05$), with a reduction by a factor of 0.81 in animals exposed to the lowest concentration. With respect to the three *gst* isoforms analyzed, only *gstr* presented significant differences. In gills from animals exposed to the highest concentration for 24 h, a significant reduction in *gtsp* concentration by a factor of 0.39 was observed in comparison with the control group and in animals exposed to the lowest concentration. A significant difference ($p < 0.05$) in gene expression of *ucp1* was observed in gills of animals exposed to the highest concentration for 24 h. In this group, gene expression increased by a factor of 14 in comparison to the control group and in animals exposed to the lowest concentration of Roundup. No significant differences were observed for other experimental times. No significant alteration was observed in gene expression of *nrf2*, *cat*, *sod1*, *sod2*, and *gclc* in the liver after Roundup exposure for 24 and 96 h ($p > 0.05$). However, *gpx1a* expression presented a significant increase by a factor of 3.23 in animals exposed to the highest concentration after 96 h compared with the control group. No

significant differences were observed in *gst* isoforms in the liver of animals from both experimental periods. No significant differences were observed for *ucp* isoforms in both experimental times.

Conclusion

The present study demonstrates that Roundup exposure causes an alteration of the antioxidant status in zebrafish gills and liver. Liver, the main detoxifying tissue against xenobiotics, appeared to be more sensitive and responsive than gills, as significant differences for ROS and/or ACAP were observed in liver tissue across all experimental times. ROS generation was not significantly altered in the present study, although a trend of reduction was observed after 24 h of exposure. ACAP presented a later response with a tendency to increase after 72 h. This tendency was confirmed with a significant increase after 96 h in animals exposed to the highest concentration. A reduction in ROS levels in liver tissue was observed after 24 h of exposure to the highest concentration; however, this reduction was observed only after 72 and 96 h of exposure to the lowest concentration. This indicates temporal response patterns between the two concentrations studied. At the highest Roundup concentration, ROS levels returned to control group values after 72 and 96 h. In the lowest Roundup concentration, this return was not observed. A reduction in ACAP levels was observed after exposure for 24 h to the highest concentration, with a subsequent increase response after 48 h. After this, ACAP concentration returned to levels similar to those observed in the control group. A concomitant reduction in ROS levels and ACAP was observed in liver tissue after Roundup exposure. ACAP levels increased significantly after 48 h of exposure, although ROS levels remained reduced. No significant differences were observed in the present study regarding *nrf2* concentration between gill and liver tissue after exposure to Roundup at any time. However, the function of this protein is associated with its cellular presence rather than with its gene expression, so the non-significant *nrf2* gene expression result does not mean that enzymes and proteins governed by this transcription factor are not regulated. A reduction in *sod2* gene expression was observed in gills after 24 h of exposure to the highest concentration. The protein isoform coded by this gene, Mn-SOD, is located in the mitochondria, the main site for ROS production. This correlates with the reduction in ROS levels, which, in turn, would be associated with a mitochondrial dysfunction caused by Roundup. Another antioxidant enzyme that presented alterations in its gene expression was GPx. This enzyme catalyzes the transformation of H_2O_2 to H_2O using a glutathione molecule as a co substrate in the process. In gill tissue, a reduction in *gpx* expression was observed in the lowest Roundup concentration; whereas in liver tissue, an induction in the highest Roundup concentration was observed. The expression profile of glutathione S-transferase isoforms was observed and in particular, the expression of *gst1a, gstp*, and *gstq* in both gill and liver tissue from zebrafish was observed. The present study produced an interesting result, wherein an increase in expression (by a factor of 14) of *ucp1* was observed in zebrafish gills exposed to Roundup for 24 h at the highest concentration. The results of the present study demonstrate that exposure to Roundup leads to a disturbance in the oxidative status of zebrafish gill and liver tissues. This effect characterizes an alteration in cellular redox state, which, in turn, would cause disturbances in cellular signaling pathways related to antioxidant response. Further, Roundup exposure interferes with genes that are not classically involved in the antioxidant defense system, such as ucp1, which, in turn, may contribute to an indirect restoration of the oxidative balance.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The data presented demonstrates that in the presence of a toxicant, there are changes in the oxidative status of zebrafish gills and liver tissue. However, these data cannot be related to an ecotoxicological regulatory risk assessment for the glyphosate EU renewal. In addition, it is not clear which Roundup formulation was exactly tested.

Further point of clarification:
The test substance was identified only as 'Roundup'. The surfactant system in the formulated product used in this study was not confirmed by the author. It is therefore not possible to confirm whether the product used, is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The objective of this study was to determine the effects of Roundup on oxidative status in adult Danio rerio liver and gills. Reactive oxygen species and antioxidant capacity were measured in fish after exposure to Roundup. Furthermore, gene expression related to antioxidant response was evaluated. These parameters are not relatable to the risk assessment given that no link with effects on individuals could be made and the study is considered not relevant.

Not relevant for regulatory risk assessment purpose given that no link between effects on gene with effects on individuals could be made.

Data point:	CA 8.2.1, CP 10.2.1
Report author	Xie RuiTao et al.
Report year	2010
Report title	The acute toxicity of five pesticides to yellow catfish Pelteobagrus vachelli.
Document Source	Fisheries Science (2010), Vol. 29, No. 5, 7 pages
Guidelines followed in study	None
Deviations from current test guideline	Not applicabel
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The acute toxicity of glyphosate to yellow catfish *Pelteobagrus vachelli* juveniles was determined in a static method at room ambient temperature. Glyphosate had the LC_{50} of 15.38 mg/L for 24 h, 13.43 mg/L for 48 h, and safe concentration of 3.072 mg/L.

Materials and Methods

Test organism
Pelteobagrus vachelli is provided by the Kejia Fish Culture Experiment Farm of Nanning City, Nanning. The average body length was 3.0 cm and the average body mass was 1.1 g. Temporarily cultured in the experimental base. During domestication, its behavior and feeding were normal. Mortality <1%. Feeding was stopped 24 hours before the test.

Test item
Glyphosate: 0.11-15 g/m^2, obtained from Chengdu.

Test item allocation
Glyphosate was added into mother liquor with distilled water, and then diluted to the required concentration. Glyphosate was tested in 5 different concentrations: 7.0, 9.11, 11.38, 15.38, and 20 mg/L (Group 1-5).

Test method
Tested by static acute toxicity test. The specification of the glass aquarium for the test is 60 cm × 30 cm × 30 cm. Each glass aquarium contained 20 L of test solution with 20 test fish for each glyphosate concentration dn the control group. 3 aquariums were used for each concentration tested. Throughout the whole test process, the aquarium was slightly inflated, DO > 5.0 mg/L, and the water temperature was maintained at (26 ± 1 °C). The fished were not fed during the test. The test solution was replaced every 12 h. The test duration was 96 h, The behavior changes of the test fish were observed and recorded during the test. The test began with 8 h continuous observation, and the test fish deaths were observed at 24, 48, 72, and 96 h, respectively.

Data processing
According to the concentration of the pesticide, the time of death, the death rate, the median tolerance limit (TLM) was obtained by linear interpolation. The safety concentration (SC) was obtained according to the Turubell formula.

The SC of the pesticide is calculated as follows:

$$SC = 0.3 \times 48\text{ h } LC_{50} / (24\text{ h } LC_{50} / 48\text{ h } LC_{50})^2$$

Results

The test results of acute toxicity of glyphosate to *Pelteobagrus vachelli* for 24 and 48 hours are shown in Table 1. No dead fish were recorded in the control group, but the number of dead fish in the five trials increased with the increase of pesticide concentration.

Table 1. Test results of glyphosate exposure to yellow catfish *Pelteobagrus vachelli*

		%Death rate					
Test item	**Test time**	**Control**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 5**
Glyphosate	24 h	0	0	10	30	50	80
	48 h	0	10	40	60	90	100
	72 h	0	20	50	60	70	100
	96 h	0	30	60	70	90	100

	Time of death	-	29	17	13	7	3.5

Conclusion

Glyphosate had the LC_{50} of 15.38 mg/L for 24 h, 13.43 mg/L for 48 h, and safe concentration to yellow catfish *Pelteobagrus vachelli* of 3.072 mg/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Acute effects on Yellow Catfish in a static 96 h test. The application method (preparation of test solution etc.) is not specified. The concentrations used is unclear, and appears to be tested in a range between 7 to 20 mg/L. No information on the test item whether it was product or active ingredient was provided. Therefore, the biological results can not be used for the ecotoxicological regulatory risk assessment and are seen as unreliable.

Assessment and conclusion by RMS:

Original report in Chinese, results are based on a document translated in english.
The acute toxicity of glyphosate to yellow catfish Pelteobagrus vachelli juveniles was determined in a static test. Glyphosate had the LC50 of 15.38 mg/L for 24 h, 13.43 mg/L for 48 h, and "safe concentration" of 3.072 mg/L. "Safe concentration" was not defined but it is certainly not a NOEC as its value is lower than the lowest tested dose.
The application method (preparation of test solution etc.) is not specified. The concentrations used are unclear (appear to be tested in a range between 7 to 20 mg/L). It is not known if they correspond to nominal or measured concentration. Clear dose-effect relationship was observed, that may be indicate a greater senvity of the catfish species. However no information on the test item whether it was product or active ingredient was provided.
The study is less relevant but supplementary but the results are seen as unreliable for regulatory risk assessment purpose.

Data point:	CA 8.2.1
Report author	Yusof S. et al.
Report year	2014
Report title	Effect of glyphosate-based herbicide on early life stages of Java medaka (*Oryzias javanicus*): a potential tropical test fish.
Document Source	Marine pollution bulletin (2014), Vol. 85, No. 2, pp. 494-498
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Java medaka adults were cultured in the laboratory and the fertilized eggs of the F2 generation were exposed to different concentrations of glyphosate-based herbicide (100, 200, 300, 400 and 500 ppm) until they hatched. The survival and hatching rates of the embryos, changes in the heart rate and morphological impairments were recorded. Generally, survival and hatching percentage decreased as glyphosate concentration increased. Absence of pectoral fin(s) and cornea, permanently bent tail, irregular shaped abdomen, and cell disruption in the fin, head and abdomen are among the common teratogenic effects observed. Furthermore, risk factor also increased with the increased in glyphosate concentrations.

Materials and Methods

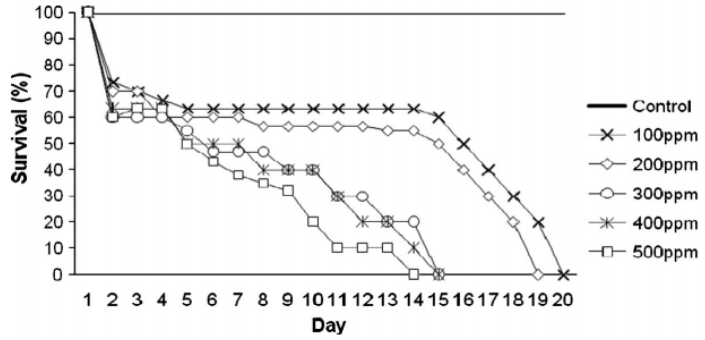
Java medaka was cultured in the Department of Biology, Universiti Putra Malaysia. Culture conditions were as follows: salinity 20 ppt, temperature 28-30 °C (ambient), pH 5.5-6.5, D.O 6.0-8.0 mg L^{-1}, and photoperiod 14 h light:10 h dark. Newly-spawned Java medaka eggs clusters from F_2 generation were collected gently from the female's body by hand. The clusters of eggs were separated using forceps and then surface sterilized using a mixture of NaCl, KCl, CaCl ×·$2H_2O$, $MgSO_4$·× $7H_2O$ and methylene blue for 5 min. The eggs were then incubated in separate test solutions containing notional concentrations of 100, 200, 300, 400 and 500 ppm glyphosate (Roundup®). Roundup® was purchased from a garden center. The formulation contains 360,000 ppm glyphosate. Maximum dose of glyphosate as recommended by the manufacturer is 500 ppm. Thus, in this study we used this concentration and several concentrations lower.
Stock solutions of 1000 ppm were prepared in distilled water, from which small aliquots were added to saline water in order to obtain desired concentration. Ninety fertilized eggs were exposed to each glyphosate concentration. Exposure was done under semi-static system where the test solutions were renewed every 24 h. Observations on the development of the embryos were made every 24 h using stereomicroscope. Death and hatching of embryos were recorded. The heart rate of each living embryos was counted. Any developmental impairment was recorded and captured. Exposure was done till three

days post-hatch. Hatched embryos were transferred to saline water of 20.0 ppt for further observation. Differences between treatments were compared using Kruskal Wallis one-way analysis of variance.

Results

Fig. 1 shows the survival rate of Java medaka embryos after exposure to different concentrations of glyphosate within 20 days. Different concentrations of glyphosate used significantly affected the survival rate ($p < 0.05$). Generally, survival rate reduced as the glyphosate concentration increased. Embryos exposed to 300, 400 and 500 ppm glyphosate were all dead by the end of the exposure period. As the survival of the embryos decreased, the hatching rate also decreased when exposure concentrations increased. Rank of hatchability based on glyphosate concentrations is as follows: control > 100 ppm = 200 ppm > 300 ppm > 400 ppm = 500 ppm. Glyphosate was found to inhibit the enzyme 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS). Only 50% of the embryos exposed to 100 ppm glyphosate died after 16 days of exposure.

Figure 1. Survival rate of early life stages of Java medaka after exposure to different concentrations of glyphosate.



The heart of the embryo started to beat at day-three post-fertilization. Normal heart rate for early life stages of Java medaka found in this study for 16 day observation was between 101 and 140 beats per minute. Exposure to glyphosate initially increased the heart beat compared to normal and it later on fluctuated and finally slowed down or halted. The highest heart rate was found in embryos exposed to 500 ppm glyphosate on the fifth day of exposure (162 beats per minute).

Several developmental abnormalities were observed in pre-hatch Java medaka embryos when exposed to different concentrations of glyphosate. The developmental impairments were shrunken egg yolk, abnormal body curvature, slow development and disproportionate head and body size. Normal incubation period for the embryos is twelve to fourteen days. Embryos that took more than fifteen days to hatch were considered as slow developing embryo. Percentage of developmental abnormalities increased as the glyphosate concentration increase. In post-hatch larvae, several developmental impairments were also observed which include absence of pectoral fin(s) and cornea, permanently bent tail, abdominal enlargement and cell disruption in the fin, head and abdomen.
Since the concentrations of glyphosate used in this study were considered high and the negative effects of the chemical have been observed in the lowest concentration used, further tests in lower concentrations are recommended to investigate the lowest observable effect level (LOEL) of glyphosate in Java medaka.

Conclusion

The survival and hatching rates of the embryos, changes in the heart rate and morphological impairments were recorded. Generally, survival and hatching percentage decreased as glyphosate concentration increased. Absence of pectoral fin(s) and cornea, permanently bent tail, irregular shaped abdomen, and cell disruption in the fin, head and abdomen are among the common teratogenic effects observed.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: There is insufficient explanation provided on the analytical verification of the test concentrations. It is not clear which Roundup formulation was tested. The test concentrations were high ranging from 100 to 500 ppm. A regulatory endpoint is not available. There is no verification of dose levels, and the study does not conform to any guidelines nor GLP.

Further point of clarification:
The test item purity is not stated in the paper. The test substance was identified only as 'Roundup'. The surfactant system in the formulated product used in this study was not confirmed by the author. It is therefore not possible to confirm whether the product used, is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with high level of caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Java medaka adults were cultured in the laboratory and the fertilized eggs of the F2 generation were exposed to different concentrations of Roundup formulation (100, 200, 300, 400 and 500 ppm) until they hatched. The survival and hatching rates of the embryos, changes in the heart rate and morphological impairments were recorded.
50% of the embryos exposed to 100 ppm glyphosate died after 16 days of exposure. Several developmental abnormalities were observed in pre-hatch Java medaka embryos when exposed to different concentrations of glyphosate. No NOEC could be derived. Results only show that high concentrations of Roundup induce developmental toxicity in Java medaka but such concentrations are far above those expected in realistic conditions.
The potential presence of surfactant that may influence the results has not been mentioned in the study.
The study is less relevant but supplementary based on RMS criteria. In this case, given the uncertainty around the impact of surfactant and mostly as the results of this study does not provide any information that can be used in the risk assessment, a detailed reliability assessment was conducted.

Data point:	CA 8.2.8
Report author	Yang X. *et al.*
Report year	2019
Report title	Effects of the glyphosate-based herbicide roundup on the survival, immune response, digestive activities and gut microbiota of the Chinese mitten crab, *Eriocheir sinensis.*
Document Source	Aquatic toxicology (2019), Vol. 214, Article No. 105243
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

The summary as proposed by the applicant is presented here.

Full summary of the study according to OECD format

In this study, the survival rate, intestinal and hepatopancreatic immune and digestive functions, and the intestinal microbial diversity of Chinese mitten crab (*Eriocheir sinensis*) were evaluated after 7 days of exposure to glyphosate (48.945 mg/L from 1/2 96-h LC_{50} value). The results showed that glyphosate significantly reduced the survival rate of *E. sinensis*. After exposure to glyphosate, the total antioxidant capacity (T-AOC) in the midgut and hindgut of *E. sinensis* was significantly decreased, and malondialdehyde (MDA) content in the midgut was significantly increased ($P < 0.05$). After glyphosate exposure, the activities of digestive enzymes (including lipase and amylase) in the intestinal tract were significantly decreased and trypsin was significantly increased, while three enzymes in the hepatopancreas were significantly increased ($P < 0.05$). Using high-throughput sequencing analysis of the gut microbiota, the results showed that glyphosate significantly decreased the diversity of *E. sinensis* gut microbiota, while significantly increasing the taxonomic richness of *Bacteroidetes* and *Proteobacteria* ($P<0.05$). This study suggested that these bacteria may be involved in glyphosate effects on survival by regulation of immune and digestive function.

Materials and methods

The experiment crabs, *E. sinensis,* were obtained from the Chongming Island base of Shanghai Ocean University. All were juvenile crabs weighing 23.49 ± 3.45 g. The crabs were kept for one week in a circulating water system. During the environmental adaptation process, the water temperature was maintained at 19–22℃, the pH between 7.6–7.8, and the dissolved oxygen level at >6.0 mg/L, and adequate aeration was maintained.

A total of 30 experimental crabs (healthy and active) were kept in blue plastic boxes (40×30 x 20 cm) and divided into a control group (C) and an experimental group (A). There were three replicates in each group and each replicate contained 5 crabs. The experimental group was treated with glyphosate solution (Monsanto, USA) in water at the half of semi-lethal concentration of glyphosate (48.95 mg/L) for 96 h. The crabs were fed 5% of their own weight, and food and feces were removed daily. Half of the experimental water was changed every day. After 7 days, the survival rate of *E. sinensis* was recorded. The hepatopancreas and intestine of each crab were collected quickly and stored at -20 ℃ for determination of antioxidant capacity and digestive enzyme activity. Total antioxidant capacity (T-AOC) and malondialdehyde (MDA) were measured in the midgut and hindgut, respectively. The activity of lipase (LPS), amylase (AMS) and trypsin (TRY) were assayed in the whole intestine (midgut plus hindgut). The contents of the hindgut from each crab were expressed into a sterile centrifuge tube, pooled, and stored at -80 ℃ for gut microbial analysis.
After thawing, the intestinal tissue was added to a centrifuge tube with a tissue: physiological saline ratio of 1:9 (weight to volume), homogenized using a homogenizer, and centrifuged at 12,000 r/min for 10 min. The supernatant was collected for subsequent enzyme activity determination. The hepatopancreas was treated as above, homogenized in a homogenizer, centrifuged at 12,000 r/min for 10 min. The middle layer was transferred to a new centrifuge tube, centrifuged again for 10 min, and finally the supernatant was collected. For the subsequent enzyme activity assay, the levels of T-AOC,

MDA, LPS, AMS and trypsin (TRY) in the hepatopancreas and intestine were determined using a kit from Nanjing Jiancheng.

Total DNA was extracted using a rapid DNA rotation extraction kit. The quality of DNA was evaluated by 0.8% agarose gel electrophoresis, and DNA was quantified by ultraviolet spectrophotometer.

Operational Taxonomic Unit (OUT) usually refers to merging sequences from one or more samples according to an artificial sequence similarity threshold. Sequences whose similarity is higher than the threshold were merged into an OTU. Based on the OTU abundance matrix, the species richness of each community was estimated, and a Venn diagram was generated to represent the number of shared and unique species and the percentage of OTUs (%) in the population.

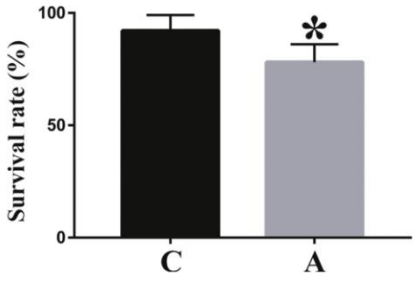
There are many indices used to quantify the alpha diversity of microbial communities. Different indices have different emphases for measuring community diversity. The main indices that reflect community richness are the Chao1 index, the ACE index, the Shannon index and the Simpson index. The main purpose of beta diversity analysis is to investigate the similarity of community structure among different samples.

Based on the known microbial genome data, the microbial metabolic function was predicted by sequencing the data for microbial community composition. By using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States), the existing 16S rRNA gene sequencing data were compared with the microbial reference genome database for known metabolic functions.

Results

The survival rate of crabs exposed to glyphosate was significantly less than that of the control group ($P < 0.05$) (Fig. 1).

Figure 1. Effect of glyphosate on the survival rate of crabs. C: the control group; A: the experiment group. The values are expressed as the means ± SD (n = 5). The asterisk above the bars indicates a significant difference ($p < 0.05$) between treatments.



For antioxidant capacity 7 days after exposure to glyphosate, T-AOC in the midgut and hindgut decreased significantly compared with the control group, and MDA content in the midgut increased significantly (Fig. 2). The activities of digestive enzymes in the hepatopancreas, lipase (LPS), amylase (AMS) and trypsin (TRY), increased significantly after exposure to glyphosate for 7 days compared with the activities in the control group (Fig. 3(2)). LPS and AMS activity in the intestine decreased significantly compared with that of the control group, whereas the opposite trend was observed for TRY activity (Fig. 3(1)).

Figure 2. Antioxidant capacity of the intestine and hepatopancreas. (1) Total antioxidant capacity (T-AOC); (2) malondialdehyde (MDA); The values are expressed as the means ± SD (n = 5). Asterisks above the bars indicate significant differences ($p < 0.05$) between the control and the experimental group.

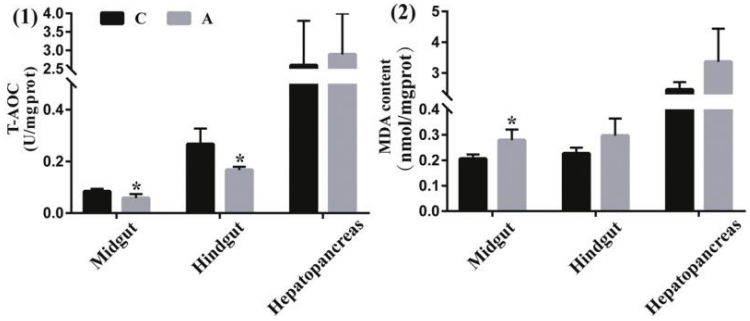
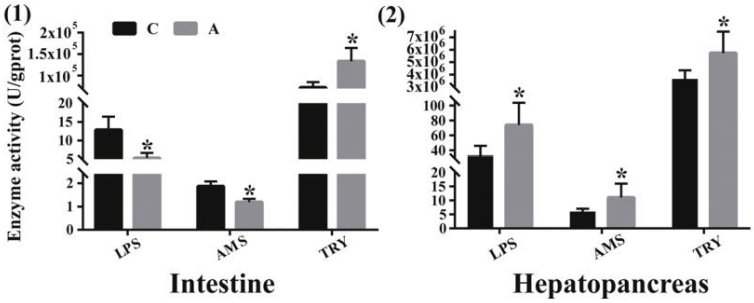
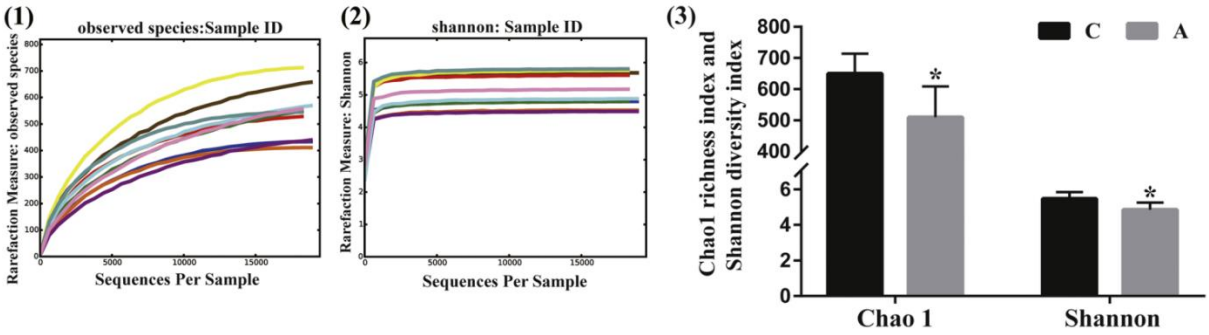


Figure 3. The activity of digestive enzymes in the hepatopancreas and intestines of crabs. (1) Digestive enzymes activity of intestine; (2) Digestive enzymes activity of hepatopancreas; The values are expressed as the means ± SD (n = 5). Asterisks above the bars indicate significant differences ($p < 0.05$) between the control and the experimental group.



An illumine HiSeq 2000 sequencing system was used to generate 343,611 effective sequences from the gut microbiota. After mass filtration, 291,804 sequences were obtained for analysis (84.92%). A total of 10,104 OTUs were classified after a 97% phase sequence similarity comparison. The reduced number of OTUs is 2444. These were characterized into different taxonomic levels. Seven days after glyphosate exposure, there were 729 overlapping OTUs between the two groups, 900 OTUs were unique to the control group (C), and 655 OTUs were unique to the experimental group (A). This result showed that the current depth of sequencing was sufficient to reflect the diversity of the tested samples. From Fig. 4(3), it can be seen that after glyphosate treatment, both the Chao 1 richness and the Shannon diversity index of gut microbiota in crabs were significantly lower than those indices in the control group ($P < 0.05$).

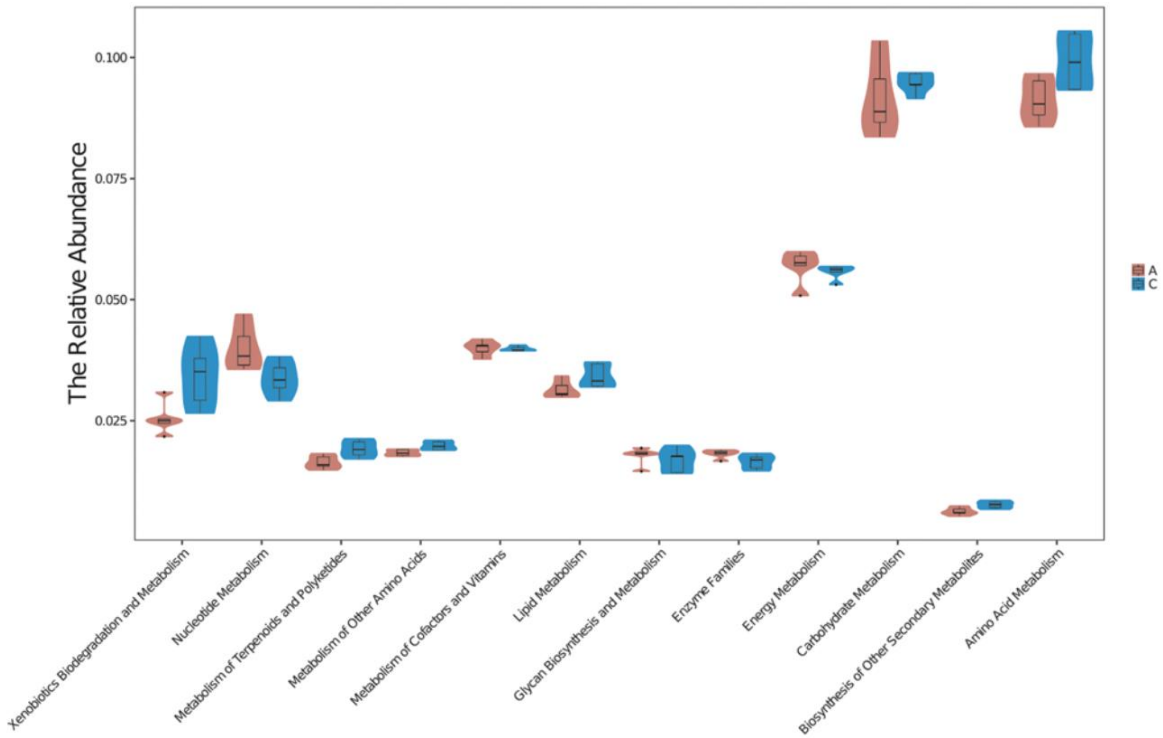
Figure 4. Effects of glyphosate on the richness and diversity of the gut microbiota in crabs. (1): Rarefaction curves; (2): Shannon curves; (3): Chao 1 and Shannon richness and diversity index for the two groups.



Due to the diversity of microorganisms, different microorganisms have specific phylogenetic relationships with each other. These results show that there are significant differences in microbial flora composition between the control group and the glyphosate exposed groups.

At the phylum level, results showed that there were no significant differences between the two groups in Tenericutes and Firmicutes abundance levels ($P > 0.05$), but the relative abundance of Proteobacteria was significantly higher in the glyphosate exposure group than in the control group ($P < 0.05$), and the relative abundance of Bacteroidetes was significantly higher in glyphosate exposure group ($P < 0.05$). The differences between the samples within each group were small, indicating that the species abundances of the control group and the glyphosate exposure group were very different. By comparing the predicted microbial function of gut contents, it was found that the relative abundances of energy, carbohydrates and amino acids in the control group and the glyphosate exposure group were significantly higher than those for other metabolic functions (Fig. 5).

Figure 5. KEGG Distribution Map Predicted by PICRUSt



Conclusion

In this study, glyphosate exposure significantly reduced the survival rate of *E. sinensis* and had a negative impact on antioxidant capacity and digestive enzyme activity. In addition, after glyphosate exposure, the diversity of intestinal microorganisms and the relative abundance of dominant bacteria in *E. sinensis* were significantly affected, but the mechanisms of these effects requires further study.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear. In addition, this study uses a high dose of Roundup formulation. The surfactants in Roundup are known to be toxic to aquatic animals. This publication indicates a potentially significant decline in survivial due to Roundup. Therefore, results obtained for other endpoints beyond survival may be secondary to known toxicity of the surfactants. The findings are not relatable to an EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Further point of clarification:
The test item purity is not stated in the paper. The test substance was identified only as 'Roundup'. The surfactant system in the formulated product used in this study was not confirmed by the author. It is therefore not possible to confirm whether the product used, is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with high level of caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

In this study, the survival rate, intestinal and hepatopancreatic immune and digestive functions, and the intestinal microbial diversity of Chinese mitten crab (Eriocheir sinensis) were evaluated after 7 days of exposure to a Roundup formulation. One tested dose: 48.945 mg/L.
The results showed that roundup significantly affected all the parameters investigated.
RMS notes the absence of clear conceptual link between effects on the digestive enzyme activity, the diversity of intestinal microorganisms and the specific protection goals for aquatic invertebrates (SPG). It is agreed that it may play a role in the population health, but such link is not immediate in conceptual terms and not quantifiable.
Data on survival were reported (for the only concentration of 48.945 mg/L) but only graphically. This parameter is relevant. From the graph it can be inferred (by RMS) a survival rate of approximately 75-80% for the treated group and above 90% in the control group. The reliability of this result cannot be verified by RMS (no detailed results). RMS highlights that the other parameters investigated in this study do not seem affected to a large extent particularly considering the high concentration that was tested. Then, the study do not provide evidence that such effects would occur under realistic conditions of use (tested concentration was far higher the RAC value). No endpoint can be derived from this study.
No specific guideline was followed, no analytical verification was available. Besides, the test substance was identified only as 'Roundup'. The surfactants were not stated. Effects (lethal and sublethal) were observed but it is not possible to discriminate between glyphosate and surfactants.
Overall, the study is considered less relevant but supplementary (formulation) but not relevant for risk assessment and not reliable.

Data point:	CA 9
Report author	Georgieva E. *et al.*
Report year	2018

Report title	Glyphosate-based herbicide alters the histological structure of gills of two economically important cyprinid species (common carp, *Cyprinus carpio* and bighead carp, *Aristichthys nobilis*)
Document No	Applied Ecology and Environmental Research (2018), Vol. 16, No. 3, pp. 2295-2305
Guidelines followed in study	APHA, 2005
Deviations from current test guideline	Not mentioned
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The present study primarily aims to investigate the histopathological effects which a glyphosate based herbicide could cause on the gills of two economically important Cyprinid fish (common carp and bighead carp), and to determine which species is more sensitive in terms of glyphosate contamination. The pesticide concentrations tested in laboratory conditions were decreasing (72 mg/L, 40 mg/L and 20 mg/L) and prepared by dilution of the stock solution of the commercial product used in plant protection. The experiment was short-term of 96 h. In general, the herbicide caused different pathological alterations in the fish gills, such as lamellar lifting, edema, proliferation of the glandular cells and epithelium, covering the gill filament, fusion, vasodilatation of the secondary lamellae and aneurysms. In addition, the researchers observed a tendency towards the enhancement of the gill histological changes which degree of expression was proportional to the increasing pesticide concentrations. However, bighead carp was more sensitive compared to common carp when it comes to the tested chemical and the alterations in the gill histological structure were more pronounced.

Materials and methods

Chemicals and experimental setup
The test pesticide in the present study was a glyphosate-based herbicide which is used to control grasses, herbaceous plants, including deep-rooted perennial weeds, some broadleaf trees, shrubs, and conifers. The researchers used decreasing glyphosate concentrations of 72 mg/L, 40 mg/L and 20 mg/L under laboratory conditions for a total acute period of 96 h. All three concentrations were prepared in advance by dilution of the commercial product stock solution used in plant protection, given by the company which produces the chemical.
Forty healthy common and bighead carps were obtained from the Institute of Fisheries and Aquaculture, Bulgaria where the fish are reared under strict controlled conditions. The fish were of the same size-group (mean length 17.6 cm ± 2.9 and mean body mass 46.3 g ± 8.4 for common carp and mean length 18.65 cm ± 1.33 and mean body mass 53.02 g ±5.3 for bighead carp) with no observed external pathological abnormalities. After transportation, the fish were moved in 100 L glass aquaria with chlorine-free tap water (by evaporation) to acclimatize for a week. They were randomly divided into four groups, including control (n=10) and not fed during the experiment.
The water physico-chemical characteristics, such as pH, temperature, dissolved oxygen, oxygen

saturation and conductivity were measured once per day according to a standard procedure (APHA, 2005). All experiments were conducted in accordance with Directive 2010/63/EU.

Histopathological analysis
The fish dissection was performed according to the procedures given in the EMERGE Protocol (Rosseland et al., 2003). The samples were placed in vials with 10% neutral buffered formaldehyde solution (pH 7.0). Then they were rinsed in tap water, dehydrated in a graded series of ethanol concentrations, cleared in xylene, embedded in paraffin wax with melting point of 54-56 ºC, sectioned to a thickness of 5-7 μm using a rotary microtome (Leica RM 2125) and mounted on sterilized glass slides. The sections were then deparaffinised, stained with hematoxylin and eosin (H&E) for histological examinations and prepared for light microscopy analysis (Romeis, 1989).

Semi–quantitative scoring
Ten paraffin sections were produced from the gills of each specimen. Each section was taken from a different location from the paraffin block, instead of in series. The histological alterations in the gill structure of each paraffin section were analyzed by observing the whole gill surface. The degree of expression was studied, including the corresponding changes in the gill surface in relation to the normal histological structure. The histological alterations in the gill epithelium were determined semi–quantitatively by using the grading system of Pierce et al. (1978) and Zimmerli et al. (2007) which the researchers combined and modified. Each grade represented specific histological characteristics and was categorized as follows:

(0) – no histological alterations which represented normal histological structure;
(1) – mild histological alterations;
(2) – moderate histological alterations;
(3) – severe histological alterations;
(4) – very severe histological alterations in the gill surface architecture.

Statistical analysis
The statistical program which was used was Graph Pad Prism 7 for Windows. In order to study the differences between the degree of expression of each histological alteration between the control and treated with different glyphosate concentrations groups, t-test was applied. P value was set at 0.05. This was possible as for each degree of expression a numerical value from 0 to 4 was given. Ten fish from each test group and species were analyzed. The results are presented as average.

Results

No histopathological changes were observed in the control fish gills. With regard to the grading system, proposed as the control carp histological characteristics, these were evaluated as relatively normal (0). Comparing both Cyprinids exposed to glyphosate, the researchers found in the gill structure proliferative changes (lamellar lifting, edema, proliferation of epithelial covering the filament and glandular cells, proliferation of cartilage, fusion), degenerative changes and changes in the circulatory system (vasodilatation of the primary sinus and blood vessels in the secondary lamellae) which all varied in their degree of expression.

Table 1 shows that lamellar lifting and edema were more pronounced – in severe and very severe degree of expression in both tested species, when increasing the concentrations of glyphosate. The researchers consider that lamellar lifting and edema actually represent a compensatory-adaptive mechanism which increased the distance between the epithelium and blood vessels in the secondary lamellae. Hence, the changes found in the fish gills could slow down the oxygen access into the blood circulatory system which in turn would result in a gas exchange limit. In addition, the researchers found proliferation of the filamentous epithelium as probably another type of compensatory-adaptive mechanism in the fish gills exposed at all herbicide concentrations. It increased the thickness and the layers of the different epithelial cells which could likely delay the toxicant access through the gills. Proliferation of the filamentous

epithelium was expressed mainly in a severe degree in the gills of common carp, while in the bighead carp gills it was found in a very severe degree of expression. Furthermore, proliferation of the connective cartilage tissue, leading to increased volume of gill filaments, was presented only in the gills of bighead carp which showed specificity in regards to glyphosate contamination. Fusion as a more severe form of proliferation of the filamentous stratified epithelium was presented in a similar degree of expression in both tested species. In terms of the proliferative changes, the researchers consider that bighead carp showed higher sensitivity under the influence of glyphosate compared to common carp because they also observed very severe proliferation of the filamentous epithelium and cartilage hyperplasia.

Table 1. Histopathological alterations in common carp and bighead carp gills caused by the test herbicide

Gills histopathological alterations	Concentration herbicide							
	Common carp				Bighead carp			
	Control	20 mg/l	40 mg/l	72 mg/l	Control	20 mg/l	40 mg/l	72 mg/l
Lamellar lifting	1	3	4	4	1	**2***	4	**4***
Edema	0	2	3	4	0	2	3	4
Proliferation of stratified epithelium	0	*3***	3	*3***	0	*2***	3	*4***
Proliferation of glandular cells	0	**1***	2	**3***	0	1	2	2
Fusion	0	**0***	1	**2***	0	**0***	1	**2***
Proliferation of cartilage tissue	0	*0***	0	*0***	0	*2***	2	*3***
Degeneration of gill epithelium	0	*0***	*1***	3	0	*2***	*3***	3
Vasodilatation of secondary lamellae	0	**0***	1	**2***	0	2	2	2
Vasodilatation of central venous sinus	0	1	1	2	0	2	2	2
Aneurysms	0	0	*2***	*3***	0	0	*0***	*0***

(0) – no histological alterations which represented normal histological structure; (1) – mild histological alterations; (2) – moderate histological alterations; (3) – severe histological alterations; (4) – very severe histological alterations of the gill surface architecture

* **Bold** – Significant differences in the degree of expression of each histological alteration for common carp and bighead carp separately after exposure to 20, 40 and 72 mg/l ($P < 0.05$)

** *Italic* – Significant differences in the degree of expression of each histological alteration between common carp and bighead carp together after exposure to 20, 40 and 72 mg/l ($P < 0.05$)

The researchers found changes in the gills circulatory system such as vasodilation in the secondary lamellae and the main venous sinus. Vasodilatation was presented in a moderate degree of expression in the bighead carp gills at all glyphosate concentrations, while this change was in such a degree in the common carp gills only at the highest glyphosate concentration (Table 1). Therefore, the researchres consider that bighead carp had higher sensitivity in relation to the pesticide effects which could be found even at the lower concentrations of the toxicant. It also enhanced the blood cell flow and increased the internal pressure of the blood vessels. However, aneurysms in the secondary lamellae were observed only in the common carp gills. Despite of the internal pressure and vasodilatation in blood vessels, aneurysms in the secondary lamellae of the bighead carp gills did not occur (Table 1).

Degenerative changes in the gills were also presented in both fish species which were found in a moderate and severe degree in the bighead carp gills at all glyphosate concentrations, while they were presented in a severe degree in the common carp gills only at 72 mg/L glyphosate (Table 1).

The observed proliferative and degenerative histopathological alterations in this study suggest the development of a two-way process in the fish gills under the glyphosate influence. On one hand, the toxicant induced a compensatory-adaptive process which was related to transport delay of the toxicant by lamellar lifting and edema, as well as induced process of mitosis in the gill epithelium and cartilage in the bighead carp gill. On the other hand, glyphosate induced degenerative changes which were related with necrotic processes. Regarding the two Cyprinids separately the t-test proved significant differences ($P < 0.05$) for the degree of expression of proliferation of glandular cells between the fish exposed to 20 mg/L and 72 mg/L. Similar differences were also proved for fusion and vasodilatation of secondary lamellae between 20 mg/L and 72 mg/L (Table 1). Regarding the degree of expression of the histological

alterations in bighead carp, the statistical analysis proved significant differences for lamellar lifting and fusion between the groups treated again with the lowest and highest glyphosate concentrations.
Comparing the two fish species together, statistically significant differences were found in the degree of proliferation of stratified epithelium in common carp and bighead carp between 20 mg/L and 72 mg/L. These were also found for proliferation of cartilage tissue and aneurysms between 20 mg/L and 72 mg/L, and for degeneration of gill epithelium but between 20 mg/L and 40 mg/L ($P < 0.05$).

Conclusion

In sum, the researchers observed proliferative and degenerative changes in the bighead carp gills at all glyphosate concentrations. However, pronounced proliferative histopathological alterations in the common carp gills were found at the highest pesticide concentration. These results showed bighead carp as a more sensitive species in comparison to common carp. Therefore, the researchers believe that bighead carp is an appropriate species for ecotoxicological tests, as well as a good bioindicator for pesticide pollution of water bodies. The researchers consider that the results from such *ex situ* experiments could be applied for better plant protection practices, in the field of water legislation for reducing maximum allowable concentrations of organic pollutants, and the protection of freshwater fauna by using bioindicators and applying histological biomarkers.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Sub-lethal effects at the histopathological level are not considered in the EU level ecotoxicological regulatory risk assessment, therefore the observations in the paper cannot be related to the glyphosate EU renewal. In addition, it is not possible to confirm the identity of the test substance used in the study as only *glyphosate-based herbicide* is mentioned. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Assessment and conclusion by RMS:

This study investigates the histopathological effects of a glyphosate based herbicide on the gills of two Cyprinid fish (common carp and bighead carp). The concentrations tested in laboratory conditions were high (72 mg/L, 40 mg/L and 20 mg/L), 96 hours.
Pathological alterations in the fish gills were observed. Bighead carp was more sensitive compared to common carp.
The test item is not well defined. No analytical verification was reported.
Fish were rather big: 17.6 cm ± 2.9 and mean body mass 46.3 g ± 8.4 for common carp and mean length 18.65 cm ± 1.33 and mean body mass 53.02 g ±5.3 for bighead carp. The study may not cover sensitivity of younger individuals.
The pH, temperature, dissolved oxygen, oxygen saturation and conductivity were measured but values were not reported.
Histopathological effects are not directly relatable to the risk assessment. Semi-quantitative scoring was used to quantify the degree of change of gill surface (this parameter is used to assess the histological alteration of the gills). These degree of severity as they are described (no effect, mild, moderate, severe, very severe) cannot be used in the standard risk assessment. RMS considers this appropriate to compare the sensitivity of the 2 species (main aim of this study) but of limited relevance for the risk assessment itself.
The concentrations tested were far above those expected in environmentally realistic conditions, so the study is not considered relevant.

Data point:	CA 9
Report author	Geyer R. L. *et al.*
Report year	2016
Report title	Effects of Roundup formulations, nutrient addition, and Western mosquitofish (*Gambusia affinis*) on aquatic communities.
Document No	Environmental science and pollution research international (2016), Vol. 23, No. 12, pp. 11729-39
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). Observations are caused by mixture of compounds / potentially causal factors and thus not attributable to a substance of concern (e.g. mixture toxicity). In addition, none of the glyphosate-based herbicides tested is the representative formulation for the glyphosate EU renewal. As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal. The surfactant system used in the formulation tested in this paper is polyethoxylene amine based, whereas the surfactant used in MON 52276 is quaternary-ammonium based.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
In this study, it is clearly stated by the study authors that the formulations contained other active substances (other than glyphosate). Besides the other compounds included very likely the surfactant polyethoxylated tallow amine (POEA). Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Therefore it is not possible to discriminate between glyphosate, the other active substances and POEA.
The study is considered not relevant by RMS (formulation containing POEA).

Data point:	CA 9
Report author	Hued A. C. *et al.*
Report year	2012
Report title	Exposure to a commercial glyphosate formulation (Roundup Max®) alters normal gill and liver histology and affects male sexual activity of *Jenynsia multidentata* (Anablepidae, Cyprinodontiformes).
Document No	Archives of environmental contamination and toxicology (2012), Vol. 62, No. 1, pp. 107-17
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The aim of this study was to evaluate the histological lesions of the neotropical native fish, *Jenynsia multidentata*, in response to acute and subchronic exposure to Roundup Max and to determine if subchronic exposure to the herbicide causes changes in male sexual activity of individuals exposed to a sublethal concentration (0.5 mg/L) for 7 and 28 days. The estimated 96-h LC_{50} was 19.02 mg/L for both male and female fish. Gill and liver histological lesions were evaluated through histopathological indices allowing quantification of the histological damages in fish exposed to different concentrations of the herbicide. Roundup Max induced different histological alterations in a concentration-dependent manner. In subchronic-exposure tests, Roundup Max also altered normal histology of the studied organs and caused a significant decrease in the number of copulations and mating success in male fish exposed to the herbicide.

Materials and methods

Adult male and female individuals of the native widespread species *J. multidentata* were used for the experiments based on their suitability for laboratory studies. The selection criteria included their small size, ease of collection and maintenance in the laboratory, and wide distribution in South America (Malabarba et al. 1998). Individuals were captured with backpack electrofisher equipment from a site on Yuspe River (648320 W; 318170 S) (Cordoba, Argentina). This site has been used as a reference location for the collection of *J. multidentata* according to previous water quality assessment studies (Hued and Bistoni 2005). Fish were transported to the laboratory in water tanks (20-L) and acclimated to laboratory conditions for 15 days before the experiments. They were maintained in a 120-L aerated glass aquarium containing dechlorinated tap water in a temperature-controlled room at 21 ± 1 °C and under a light-to-dark cycle of 12 h:12 h. During the acclimatization period fish were fed twice a day with commercial fish food. Male and female fish standard lengths (means ± SDs) were 36.34 ± 4.16 and

43.71 ± 7.46 mm, respectively. The mean weight was 0.58 ± 0.21 g for male fish and 1.12 ± 0.5 g for female fish.

Short-Term Toxicity Testing
A short-term (96-h) static toxicity tests were performed to evaluate the toxicity of Roundup Max to *J. multidentata*. Commercial glyphosate formulation was Roundup Max granular (Monsanto, Argentina) which contains 74.70% of N-(phosphonomethyl) glycine as the active ingredient and 25.30% as surfactants. Fish were exposed to the following nominal concentrations of Roundup Max: 5, 10, 20, 35, 60, and 100 mg/L for 96 h. Each control group and concentration tested was performed in duplicate with eight individuals (four male and four female fish) per group in 18-L aerated glass aquaria. Individuals were starved 24 h prior to the experiment and were not fed during the experiment.

Subchronic Toxicity Testing
For the subchronic toxicity test, two groups of nine individuals (five male and four female fish) were exposed to a 10% of the lowest concentration used in the acute toxicity test (0.5 mg/L of Roundup Max) for 7 and 28 days. Fish were fed twice a day with commercial fish food. Water of each aquarium was renewed partially every 2 days and completely removed once a week.

Male Sexual Activity
After the period of subchronic exposure, each male fish was introduced into a 10-L aquarium with an unexposed sexually mature female fish. Male sexual activity was registered using a digital camera (model no. DSC-W70; Sony) and by direct observation for 20 min with observations starting 10 min after the couple was introduced into the tank. Based on the normal reproductive behavior described by Bisazza et al. (2000), the following parameters were estimated:

1. Number of persecutions (NP): number of times that a male fish persecutes a female fish to make contact with the female gonopore.
2. Copulation attempts (CA): number of times that a male fish enlarges its gonopodium to make contact with the female gonopore.
3. Number of copulations (C): number of times that a male fish made direct contact through its gonopodium with the female gonopore.
4. Mating success (MS): estimated from the abovementioned parameters through the following formula: MS = log C/(log NP + log CA); this estimate gives an idea of the effectiveness of a male fish to copulate after persecution or copulation attempts.

Histological Analysis
After 96 h of exposure, the surviving fish, as well as those male and female fish exposed to the sublethal concentration for 7 and 28 days, were killed with an overdose of tricaine methane sulfonate, MS-222 (Sigma-Aldrich) and dissected. Gills and liver from control and exposed fish were removed and fixed in 10% formaline. Tissue samples were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin. Sections 4 to 6–lm thick were stained with hematoxylin and eosin (H&E). Tissue lesions were examined with a light microscope and photographed with a digital camera (model no. DSC-W70; Sony).

Statistical Analysis
Data distributions were analyzed using the Shapiro-Wilks index. The 96-h LC_{50} values between sexes were assessed with Student t-test. To compare the biological parameters among different Roundup concentrations and time of exposure, Kruskal-Wallis test was performed and followed by a Dunn's multiple comparison test. Differences were considered significant at $p<0.05$. Statistical analyses were performed using Infostat Software Package (Infostat 2002).

Results

Toxicity Tests: 96-h LC_{50} and Subchronic

The Roundup Max 96-h LC_{50} value for *J. multidentata* estimated was 19.02 mg/L and showed no differences between sexes. No mortality occurred in the control group or at 5 mg/L Roundup Max. All of the individuals exposed to 60 and 100 mg/L Roundup Max died during the exposure period. No mortalities were observed during the subchronic tests.

Male Sexual Activity

A significant low number of copulations (Cs) was registered in male fish exposed to 0.5 mg/L Roundup Max for 7 and 28 days with respect to the control group (Table 1). There were no differences in number of Cs between male fish exposed to 7 and 28 days to Roundup Max. MS differed significantly among treatment group and between treatment and control group, showing the lowest values after 28 days of exposure.

Table 1. Parameters of male sexual activity of *J. multidentata* exposed to a sublethal concentration of Roundup Max for 7 and 28 days

Parameters of male sexual activity	Control	7 days	28 days
NP	74.00 ± 40.00	52.40 ± 28.90	56.16 ± 29.80
CA	30.00 ± 28.00	16.00 ± 10.00	19.00 ± 9.80
C	3.31 ± 1.50	1.40 ± 0.89 (a)	1.30 ± 0.81 (a)
MS	0.17 ± 0.03	0.13 ± 0.07 (a)	0.11 ± 0.07 (b)

Values are expressed as means ± SDs. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$)

NP number of persecutions, *CA* number of copulation attempts, *C* number of copulations, *MS* mating success

Gill Histopathological Analysis

No recognizable changes were observed in gills of control individuals of *J. multidentata*. However, treated groups exposed to different concentrations of Roundup Max showed several pathological changes. The severity and frequencies of histological alterations increased with increasing concentrations used.

Liver Histopathological Analyses

Histology of control fish liver showed a normal typical paranchymatous appearance. The hepatocytes were homogeneous both in size and cytoplasm density, whereas livers of exposed fish presented different alterations, which varied in severity.

Gill Morphometric Analyses

Gill morphometrics measured presented different patterns of variation according to Roundup Max concentrations tested (Table 2). The increased severity of gill alterations was measured through increased SLW and decreased ID and SLL values.

Table 2. Gill morphometric parameters and PAGE index from acute and subchronic exposure to Roundup Max

Test	Treatment	SLL	SLW	ID	BET	PAGE
Acute toxicity test	Control	53.48 ± 3.67	8.73 ± 0.6	18.14 ± 0.53	30.18 ± 7.91	64.91 ± 6.53
	5 mg/l	59.46 ± 6.51	11.98 ± 1.22 (a)	9.16 ± 2.35 (a)	37.57 ± 7.65	60.33 ± 5.57
	10 mg/l	56.36 ± 3.81	12.89 ± 1.12 (a)	8.47 ± 2.24 (a)	38.03 ± 6.62	59.93 ± 2.56
	20 mg/l	44.48 ± 8.26 (a)	14.27 ± 0.82 (b)	7.67 ± 2.41 (a)	40.99 ± 7.5	52.02 ± 3.74 (a)
	35 mg/l	46.52 ± 0.81 (a)	13.69 ± 0.18 (b)	5.41 ± 2.42 (b)	47.84 ± 16.11 (a)	50.59 ± 8.22 (a)
Subchronic toxicity test (0.5 mg/l)	7 days	45.64 ± 11.24	10.80 ± 4.86	18.91 ± 5.54	38.13 ± 8.29	54.00 ± 6.06 (a)
	28 days	45.96 ± 9.07	8.18 ± 2.43	15.20 ± 5.48	41.20 ± 6.38	52.33 ± 2.89 (a)

Values are expressed as means ± SDs. Different letters indicate significant differences among treatments and between treatments and control group ($p < 0.05$)

SLL secondary lamellar length, *SLW* secondary lamellar width, *ID* interlamellar distance, *BET* basal epithelial thickness

Conclusion

The authors state that the research showed that acute and subchronic exposures to Roundup Max induced a diversity of gill and liver histological alterations in the neotropical fish *J. multidentata* that might impair normal organ functioning and also caused decreased sexual activity in male fish exposed to the herbicide for 7 and 28 days. The use of histopathological indices allowed quantification of histopathological lesions and thereby determination of the degree of damage in fish exposed to different concentrations of the herbicide. The registered responses could be useful indicators of toxicity by glyphosate in the studied species

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). Enzyme, cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological regulatory risk assessment. In addition, Roundup Max is not the representative formulation for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The aim of this study was to evaluate the histological lesions of the neotropical native fish, Jenynsia multidentata, in response to acute and subchronic exposure to Roundup Max and to determine if subchronic exposure to the herbicide causes changes in male sexual activity of individuals exposed to a sublethal concentration (0.5 mg/l) for 7 and 28 days.
It has been shown that the sexual activity of males was affected. RMS considers this is relevant parameter.

Roundup Max granular (Monsanto, Argentina) was used, containing 74.7 % Glyphosate and 25.3 % surfactants (likely POEA). Therefore it is not possible to discriminate between effects due to glyphosate and the ones due to surfactants. The presence of POEA was also assumed in RAR 2015. The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable.
There was no analytical verification and no measurement of water quality parameters.
Overall the study is considered not reliable.
The study is considered not relevant and not reliable by RMS.

Data point:	CA 9
Report author	Lanzarin G. A. B. *et al.*
Report year	2019
Report title	Dose-dependent effects of a glyphosate commercial formulation - Roundup® UltraMax - on the early zebrafish embryogenesis.
Document No	Chemosphere (2019), Vol. 223, pp. 514-522

Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Enzyme, cellular and molecular level endpoints are discussed that are difficult to relate to the EU level ecotoxicological regulatory risk assessment. In addition, the glyphosate formulation tested Roundup® UltraMax (35.5 wt% of glyphosate, formulated using potassium and the adjuvant ether amine ethoxylate) is not the representative formulation for the glyphosate EU renewal. Therefore, the article is not relevant for the renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The objective of this study was to investigate the lethal and sub-lethal developmental effects, neurotoxic potential and oxidative stress responses of zebrafish embryos to Roundup® Ultramax (RU) exposure.
It is stated in the study that Roundup® UltraMax contains ether amine ethoxylate (6 %).
Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product.
Besides Avigliano L. et al., 2014 (other study) also stated that (based on a comparison between Roundup Ultramax and glyphosate at equivalent concentration), Roundup Ultramax compounds other than glyphosate may be responsible for the effect of the formulation.
The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable. Therefore RMS considers that this study is of low reliability.
The study is considered not relevant.

Data point:	CA 9
Report author	Lopes da Silva E. T. *et al.*
Report year	2016
Report title	Lethal concentration of glyphosate for juveniles of Curimatã-pacu. Original title: Concentracao letal do glifosato para juvenis de Curimatã-pacu.

Document No	Boletim Do Instituto De Pesca (2016), Vol. 42, No. 4, pp. 759-764
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as provided by the applicant is presented.

Full summary of the study according to OECD format

This study aimed to determine the lethal concentration of glyphosate (LC_{50}) for juveniles of curimatã-pacu, *Prochilodus argenteus*. The experiment was conducted in CODEVASF-Três Marias, MG, Brazil, during 96 h, using 240 juvenile fish (standard length 6.4 ± 8.47 cm and weight 8.98 ± 3.91 g) The animals were submitted to six herbicide concentrations: 0, 5, 10, 15, 20 and 40 mg/L in a completely randomized design with four replications. Every 24 h the behavior of the animals and mortality rate were observed, and at 48, 72 and 96 h, the LC_{50} were estimated . The LC_{50} of glyphosate at 48, 72 and 96 h were of 20, 88; 19.91 and 19.09 mg/L, respectively. The herbicide caused the fish to swim in erratic fashion.

Materials and methods

240 juvenile curimatã-pacus were used in the experiment, with a standard length of 6.4 ± 8.47 cm and weight of 8.98 ± 3.91 g. The fish were acclimated in the laboratory for a period of seven days (APHA, 2012), in a rectangular tank (120 L), and fed twice a day with a commercial ration extruded with moisture (max). 10%, crude protein (min.) 50%, ether extract (min.) 4%, fibrous matter (max). 6%, mineral matter (max). 18%, calcium (max). 5%, phosphorus (min.) 0.15%, according to the manufacturer's specifications. Feeding was halted 24 h before the beginning of the experiment, in accordance with the recommendations of the testing standards for acute toxicity with fish, from the Brazilian Association of Technical Standards (ABNT, 1993).
The juvenile curimatã-pacu were distributed in 24 containers with a capacity of 10 L, at a density of 2.5 juveniles L_{-1}, in a static system, with constant aeration and natural photoperiod. The animals were submitted to six concentrations of the herbicide glyphosate: 0, 5, 10, 15, 20 and 40 mg/L in its commercial formulation Atanor (whose composition is isopropylamine salt of glyphosate (48% w/v; equivalent in glyphosate acid, 36% w/v) and concentration of inert ingredients, 67.9% w/v), for a period of 96 hours in a completely randomized design, with four replicates each.
The physicochemical parameters of the water: dissolved oxygen (mg/L), conductivity (μS/cm), pH, turbidity (NTU) and potential of organic redox reaction (mV), were measured by means of the YSI Professional Plus probe; ammonia (mg/L), alkalinity (mg/L) and hardness (mg/L $CaCO_3$), being carried out according to the methods specified in the "Standard Methods for the Examination of Water and Wastewater", published by the "American Public Health Association (APHA, 2012). The number of individuals of each experimental unit was measured every 24 hours so as to obtain of LC_{50} and survival rates, when the behavior of the subject was also observed. The average lethal concentration of product to 50% of the subjects (LC_{50}) was estimated using the software Trimmed Spearman-Karber 1.5 (HAMILTON et al., 1977). The assessment of the concentration levels of glyphosate on survival was

assessed by applying linear regression (P<0.05), with the aid of software SigmaStat 3.5.

Results

The water quality parameters remained, on average, with 9.7 ± 0.3 mg/L for dissolved oxygen, 6.1 ± 0.1 µS/cm for conductivity, pH of 6.8 ± 0.2, average temperature of 22.5 ± 0.3 °C. Total ammonia was lower than 0.5 ± 0.0 mg/L, alkalinity was at 21.7 ± 0.6 mg/L and hardness at 17.8 ± 0.4 mg/L $CaCO_3$. The exposure to Atanor provided a lethal concentration (LC_{50}) at 48, 72 and 96 hours of 20.88; 19.91 and 19.09 mg/L, respectively. The increase in the concentration of glyphosate led to a progressive reduction in the rate of survival of curimatã-pacu. Starting from the minimum concentration of glyphosate at 0.5 mg/L, all juveniles were initially agitated, followed by an increase in the beat of the operculum and later they showed to be swimming in erratic fashion and with lethargic behavior.

Conclusion

The LC_{50} at 96 hours of exposure to glyphosate for curimatã-pacu in the reported experimental conditions was of 19.09 mg/L, which has it as slightly toxic, according to standards of classification of IBAMA (1987).

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). The formulation tested (Atanor) is not the representative formulation for the glyphosate EU renewal. Thus the article is not relevant for the renewal.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The objective of this study was to determine the LC50 of the herbicide glyphosate (LC50) for the curimatã-pacu fish, Prochilodus argenteus.
240 juvenile curimatã-pacu fish (standard length of 6.4 ± 8.47 cm; and weight of 8.98 ± 3.91 g) were used.
The LC50 of glyphosate at 48, 72 and 96 h were of 20.88, 19.91 and 19.09 mg/L, respectively.
This study may indicate higher sensitivity of this species but the co-formulants in Atanor are not stated and may have impacted the toxicity of the product. The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable.
Moreover, no biological data and no analytical verification are presented in the report. No control data is reported. The report also states that from the minimum concentration of glyphosate at 0.5 mg L^{-1}, all juveniles were initially agitated, followed by an increase in the beat of the operculum and later they showed to be swimming in erratic fashion and with lethargic behaviour. Progressive turbidity of the water was observed and also the presence of air bubbles, leaving it with a foamy appearance. This questions the conditions of the fish in the test.
The study is less relevant but supplementary (due to the different formulation that was tested) and not reliable.

Data point:	CA 9
Report author	Lyons M. *et al.*
Report year	2018
Report title	Effects of 4-nonylphenol and formulations of five pesticides: cypermethrin, deltamethrin, glyphosate, imidacloprid and mancozeb on growth of Atlantic salmon (*Salmo salar L.*) during parr-smolt transformation.
Document No	Canadian Technical Report of Fisheries and Aquatic Sciences (2018), Vol. 3265, pp. 1-42
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

This study has shown that short term, freshwater exposure of Atlantic salmon (*Salmo salar L.*) to some pesticide formulations during parr-smolt transformation (PST) caused significant reduction of specific growth rate of fish compared to the control fish over the course of a critical seawater growth period. Effects of 4-nonylphenol and glyphosate-based herbicide Roundup WeatherMax® were tested. Both chemicals tested affected fish growth in seawater. Other studies link poor growth near PST to reduced survival at sea and lower returns of adult salmon to their native streams.

Materials and Methods

Test organisms
Atlantic salmon smolts (30-80 g) were anaesthetized in 50 ppm MS222 (Tricaine Methane Sulphonate) and individually tagged with passive integrated transponder (PIT) tags (AVID Canada, Calgary, Alberta). Fork length and weight of fish were recorded. Fish were randomly distributed into fiberglass tanks (400 L, 40 fish per tank) and allowed to acclimate in dechlorinated St. Andrews, New Brunswick municipal water at ambient temperature prior to treatments. The flow rate was maintained at approximately 5 L/min and photoperiod was regulated to simulate natural photoperiod. Except on treatment and sampling days, the fish were fed a 2.5 or 3 mm dry pellet diet from Skretting, Bayside, New Brunswick. Protocols for all experiments were approved by DFO regional animal care committee.

Test item:
The glyphosate formulation tested was Roundup WeatherMax® (Monsanto Canada Inc, Winnipeg, Manitoba). P-nonylphenol (4-NP) (Eastman Kodak Co, Rochester, New York) was used as a positive control.

Treatments:
In May of each year, the tanks were randomly assigned to be one of two replicate tanks, of one of the treatments (i.e. control, 4-NP, three concentrations of pesticide). The flow of dechlorinated water into

each tank was adjusted to 4 L/min. The fish were exposed to water-borne pesticide, 4-NP or no treatment (controls) in freshwater. The exposures took place when the freshwater temperature reached approximately 7°C. The 4-NP was dissolved in 10% ethanol/dechlorinated water. Two replicate tanks were treated with an environmentally-relevant (Fairchild et al., 1999) nominal concentration of 4-NP (20 µg/L). The pesticide formulation was dissolved in dechlorinated water and replicate tanks were treated with three concentrations. The 4-NP treatments were delivered continuously in two 24 h pulses on day 1 and 7. The pesticide treatments were delivered continuously in two 6 h pulses on day 1 and 7. The treatments were started by adding the appropriate amount of test solution required to bring each tank up to the desired concentration. Mariott bottles of the test solutions were calibrated to gravity-feed the treatment solutions at a flow rate of 1 mL/min directly into the incoming water supply of each treatment tank to ensure complete mixing. Beginning 2-3 weeks after the onset of treatments, the smolts were gradually acclimated to filtered sea water (Brandy Cove, St. Andrews, New Brunswick) over a period of 3-4 days. Seawater flow rates were maintained at approximately 5 L/min

Sampling
Pre-treatment sampling (one fish per tank) occurred on the day before the first treatment. Post-treatment sampling (four or five fish per tank) occurred for most treatments in May/June and for all treatments on two occasions, July and November (five fish per tank). Fish were stunned with a sharp blow to the head and blood was collected from the caudal vein with a heparinized disposable syringe and centrifuged at 3500x g for 10 minutes at 4°C. Plasma was aliquoted and stored at -80°C. Gill, liver, muscle and brain tissue were taken and stored for biochemical analyses. Tissues were flash frozen in liquid nitrogen and stored at -80°C. Length, weight, liver weight, blood volume and sex were recorded for each fish. After the July sampling, length and weight were recorded for all remaining fish in treatment tanks and an equal number of fish from each tank were randomly assigned to one of two large tanks (3000 L) for long-term seawater grow-out. In September length and weight were recorded for all fish. After the November sampling, remaining fish were sacrificed with an overdose of anaesthetic (MS-222) and length, weight and sex were recorded.

Heat shock protein determination
Heat shock protein 70 analysis was performed on liver samples according to Rendell et al. (2006). Briefly, ground liver was added to 15 µl homogenization buffer per mg of tissue to extract soluble protein. Samples were homogenized and centrifuged at 14000x g for 10 minutes in a Sorvall RC6 Plus centrifuge at 4°C. The supernatants were stored at -80°C after being aliquoted into two microcentrifuge tubes, one for total protein quantification and one for heat shock protein quantification.

Na^+, K^+-ATPase determination
Salmon gill samples (2007) were collected, homogenized, extracted, and analyzed using the microplate (BioTek Powerwave XS plate reader) methodology outlined by McCormick (1993). Due to the difficulties in collecting consistent gill samples, resulting in multiple extract dilutions for protein analysis, the tissue collection and extraction technique for subsequent years was a modification of an extraction proposed by Zaugg (1982). Protein levels in the same samples and standards (BSA) were quantified by UV absorbance at 600 nm on a microplate using a Bio-Rad commercial assay reagent based on the Bradford method.

AChE determination
Brain tissue collected from fish exposed to pesticides that act by inhibiting acetylcholinesterase activity was analysed to determine enzyme activity according to Ellman et al. (1961), modified by Zinkl et al. (1987). Enzyme activities were determined at room temperature using a 1:2 brain homogenate in 0.1 M Tris buffer, pH 8.

Specific growth rates
The specific growth rates based on weight (SGRW) were calculated for the time period between March PIT tagging date and July, September or November date where all available fish were measured. The SGRWs were calculated using the following formula: SGRW= ((logeY2 - logeY1)/ (t2 - t1)) * 100

where Y2 is the weight in July, September or November, Y1 is the weight in March, t2 is the day of year in July, September or November, t1 is the day of year in March.

Water analyses
Water samples (150 ml in high density polyethylene (HDPE) containers) from control and glyphosate treatment tanks were taken at T=2.5 h during the 6 h treatment (n=4). A blank water sample was taken from the dechlorinated water line. Glyphosate water samples were analysed at Natural Resources Canada, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada.

Statistical analysis
Weights and specific growth rates based on weight (SGRW) were used for statistical analysis. A non-parametric one-way analysis of variance test (NPAR-1-ANOVA) followed by a Dunnett's multiple comparison test was used to compare the mean weights and SGRWs of the control smolts with those of 4-NP and pesticide-treated 14 smolts.

The data sets used for the 2007 weight and SGRW analysis comprised of all controls, glyphosate and treated smolts available in July (n = 55 – 71), September (n = 42 – 59) and November (n = 14 – 15).

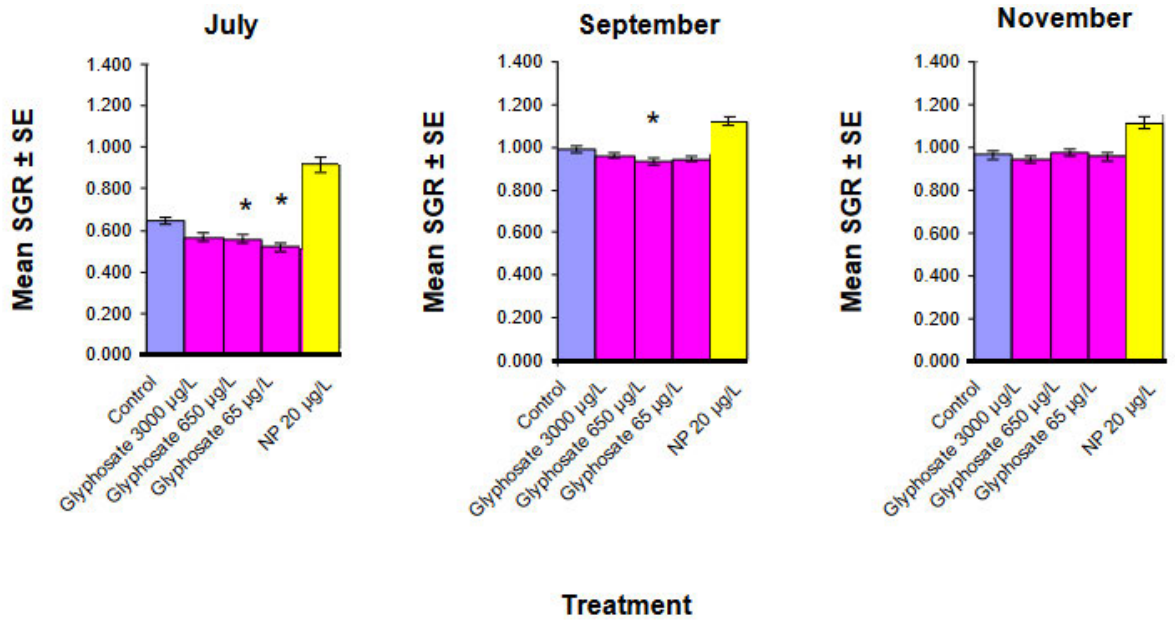
Results

A summary of comparisons of mean SGRWs between control and pesticide treatments shows there was significant difference between some treatments and controls. Comparisons between the mean SGRWs of control and treated smolts showed that SGRWs were different in July, for 15 glyphosate at both 650 µg/L and 65 µg/L. In September, glyphosate at 650 µg/L, showed significant effects in comparison to controls. By November, there were no significant differences between mean SGRWs of control and pesticide treated fish. (Dunnett's, $P < 0.05$). (Table 1 and Fig. 1).

Table 1. Difference between mean control SGRW and treatment SGRW. Asterisks represent significant difference (* $P < 0.05$, ** $P < 0.005$) (Dunnett's test).

Treatment	July	September	November
Glyphosate 3000 µg/L			
Glyphosate 650 µg/L	*	*	
Glyphosate 65 µg/L	*		
Nonylphenol 20 µg/L			

Figure 1. Mean SGRW and standard errors of control, NP and pesticide treated fish (July, September and November). Asterisks indicate significant differences from controls (P< 0.05) (Dunnett's test).



A summary of comparisons of frequency distributions of control and pesticide treatment SGRWs shows there was significant difference between some treatments and controls (Table 2). July SGRW frequency distributions (Fig. 2) and frequency histograms (Fig. 3) for glyphosate and show how SGRWs of the treated smolts were affected relative to the controls. July SGRW frequency distributions of glyphosate (3000 µg/L and 650 µg/L) treated smolts were significantly different from controls ($P < 0.05$) (KS test). July SGRW frequency distributions of glyphosate (65 µg/L) treated smolts were significantly different from controls ($P < 0.005$) (KS test). September SGRW frequency distributions of glyphosate (650 µg/L), treated smolts were significantly different from controls ($P < 0.05$) (KS test) HSP 70 Post treatment (1 to 3 days) liver samples from May and June were analysed for hsp70 induction. Exposure to 6 h pulse sublethal levels of glyphosate did not induce hsp70 in Atlantic salmon liver. Under control conditions there was no hsp70 detected in the liver. Hsp70 was detected in samples of liver from heat shocked Atlantic salmon that were loaded onto each gel as positive controls. 17 GILL NA^+ , K^+-ATPASE post treatment gill samples from July were analysed for Na^+ , K^+-ATPase activity.

Table 2. Difference between frequency distributions of control SGRW and treatment SGRW. Asterisks represent significant difference (* $P < 0.05$, ** $P < 0.005$) (KS test).

Treatment	July	September	November
Glyphosate 3000 µg/L	*		
Glyphosate 650 µg/L	*	*	
Glyphosate 65 µg/L	**		
Nonylphenol 20 µg/L	** (for 2008)	* (for 2008 & 2010)	

Water analysis Measured water concentrations for pesticide and NP treatments are shown in Table 3. These measured concentrations were generally lower than nominal concentrations. Lower measured concentrations are probably due to uptake by tubing, glassware, the surface of the tanks and the fish. Control tank water samples analysed did not show any detectable levels of pesticide.

Table 3. Water analysis-nominal and measured concentrations (data represent mean value ± SE). *average of only two analyses; ** Contract lab encountered analytical problems and no concentrations were determined.

Treatment	Measured Conc. ± SE
Glyphosate 3000 µg/L	1635 µg/L ± 129
Glyphosate 650 µg/L	714 µg/L ± 114
Glyphosate 65 µg/L	32 µg/L ± 5
Nonylphenol 20 µg/L	7.37 µg/L ± 0.94

Figure 2. July SGRW frequency distributions of controls and glyphosate treated smolts. Tick marks on the X-axis are the upper-limit of each control SGRW quartile. Y-axis gives the frequency defined by the control SGRW quartiles. Asterisks represent significant difference from the control SGRW frequency distribution (* P < 0.05, ** P < 0.005) (KS test).

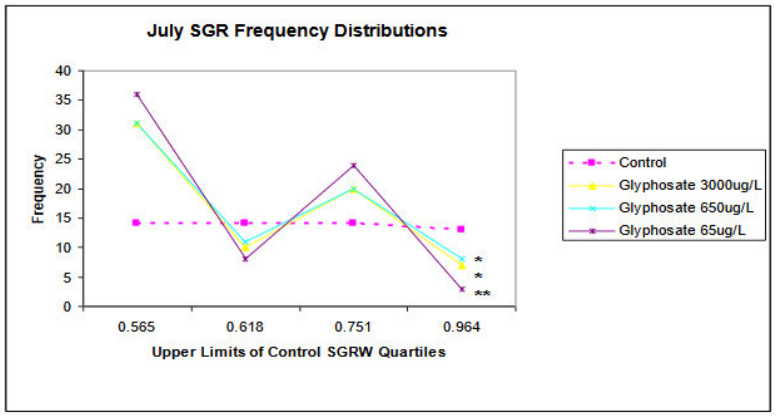


Figure 3. July SGRW frequency histogram of glyphosate treated smolts. Dotted line represents the median SGRW (i.e. 0.618) of controls.

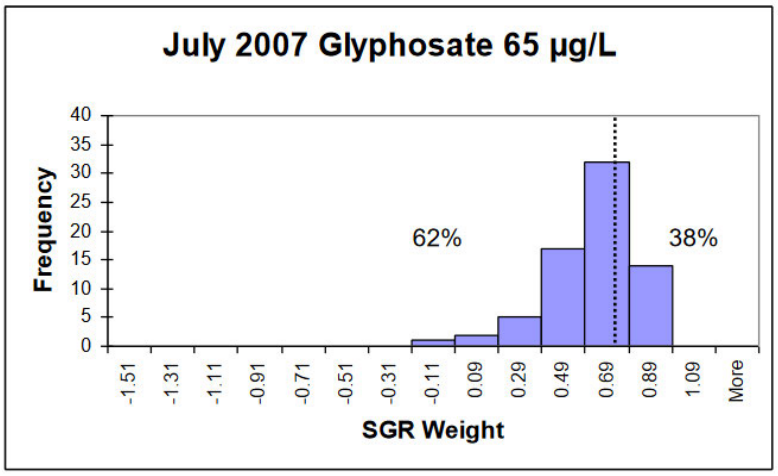
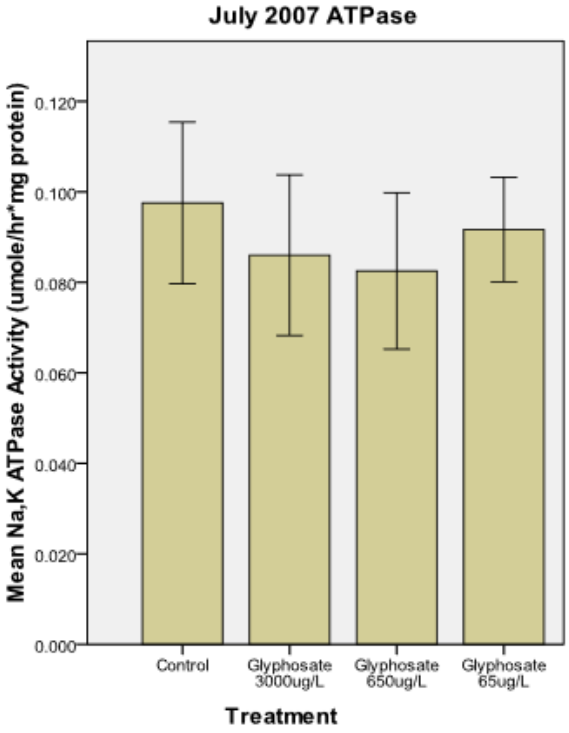


Figure 4. Mean Na, K, ATPase and standard errors of control and pesticide treated fish. Asterisks indicate significant differences from controls (Dunnett's) (P< 0.05).



Conclusion

The study has shown that short term, freshwater exposure of Atlantic salmon to some pesticides during the PST caused a significant reduction of specific growth rate of fish compared to the control fish over the course of a critical seawater growth period. Chemicals tested affected fish growth at either the July or September sample times. It remains unclear if the growth response observed in Atlantic salmon smolts is specific to the chemical being tested or is a generalized response to stress, in this case chemical stress. None of the compounds studied affected growth at all sampling times. The rate of growth picked up after an initial slow-down. Smaller fish in the wild soon after the PST may be at greater risk to decreases in disease resistance, reproductive success, smolting and swimming performance (Iwama et al., 2004). Baldwin et al. (2009) used models to show that 24 environmentally realistic pesticide exposures may limit the recovery potential of salmon populations via delayed reductions in growth and survival. The negative effects on growth observed at environmentally relevant concentrations of glyphosate are ecologically significant. These results suggest that wild Atlantic salmon smolts may be affected by pesticide runoff into rivers supporting sea-run salmon stocks. The consequences of reduced growth may be failure to survive or thrive at sea. The growth effects observed on fish exposed to glyphosate at a concentration equal to the freshwater interim guideline value of 65 µg/L evoke particular concern for the survival of wild Atlantic salmon smolts. The negative effects on growth after short-term pulse doses of waterborne glyphosate may be used to re-evaluate risk. The pesticide used in this study was a commercial formulation that contain other products to enhance the efficacy of the pesticides. These products and the possible degradation products may play a role in the negative growth affects seen.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). Atlantic salmon smolts were exposed to a glyphosate-based formulation, Roundup WeatherMax, that is not the representative formulation for the glyphosate EU renewal.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The aims of this study were to examine the effects of a glyphosate formulation Roundup WeatherMax (and 5 other products containing other substances) on growth performance of Atlantic salmon smolts. Sub-lethal cellular effects using biochemical endpoints were also investigated. This study did not follow a standardized guideline.

The growth effects observed on fish exposed to glyphosate formulation at an environmentally realistic concentration of 65 µg/L (measured 32 µg/L ± 5) evoke particular concern for the survival of wild Atlantic salmon smolts.
Mean Na+, K+-ATPase activity showed decreases in treated fish but the differences were not significant.
RMS considers the growth rate as the main parameter of interest in this study. While acknowledging that a lower growth may have temporarily affected the smolts (significant at lowest concentration), RMS notes that this was not concentration related (no significant effect at the highest concentration 3000 µg/L).
No biological data was presented, only graphs. It is difficult to explain the high difference of growth rates between the control groups used for the different experiments (control groups for cypermethrin experiments were very high compared to those of glyphosate experiments).

No biological observations are reported.

Roundup WeatherMax was used. The presence of toxic surfactants were not precised. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product (as also mentioned by the study authors concerning the formulation they used). The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable.

Therefore RMS considers that this study is less relevant but supplementary (due to the different formulation tested) and not reliable.

Data point:	CA 9
Report author	Ma Junguo *et al.*
Report year	2015
Report title	Alteration in the cytokine levels and histopathological damage in common carp induced by glyphosate.
Document No	Chemosphere (2015), Vol. 128, pp. 293-8

Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The acute toxicity of glyphosate on common carp was first determined; then, the contents of interferon- γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) and histopathological alterations in the liver, kidneys, and spleen of common carp exposed to 52.08 or 104.15 mg/L of glyphosate for 168 h were also determined and evaluated. The results of the acute toxicity tests showed that the 96 h LC_{50} of glyphosate for common carp was 520.77 mg/L. Moreover, sub-acute exposure of glyphosate altered the contents of IFN-γ, IL-1β, and TNF-α in fish immune organs. For example, there was a remarkable increase in the IFN-γ content in the kidneys, while there was a decrease in the liver and spleen. The IL-1β content increased in liver and kidneys, but it decreased in the spleen, and TNF-α mainly increased in the fish liver, kidneys, and spleen. In addition, glyphosate-exposure also caused remarkable histopathological damage in the fish liver, kidneys, and spleen.

Materials and methods

Glyphosate, kits, and chemicals
Glyphosate was purchased from Anyang Anlin Agrochemical Co., Ltd., China as a commercial formulation (50% soluble powder). Glyphosate was first dissolved in distilled water to generate a stock solution and then diluted to obtain the experimental concentrations with dechlorinated tap water.
The kits for assaying interferon-γ (IFN-γ) (Catalogue No. DRE96157), interleukin-1β (IL-1β) (Catalogue No. DRE96017), and tumor necrosis factor-α (TNF-α) (Catalogue No. DRE96155) were purchased from the Wuhan ColorfulGene Biological Technology Co., LTD, China. The other reagents used in this study were purchased from Sigma (St. Louis, USA) and were analytical grade.

Fish
Common carp (8.14 ± 1.37 g of mean body weight) were originally obtained from a local fish farm (Feilong aquarium fishery, Xinxiang, China). The fish were subjected to a prophylactic treatment by bathing twice in 0.05% potassium permanganate for 2 min before they were raised in a 200-L tank under laboratory conditions for at least two weeks before the test. The characteristics of the water were as follows: dissolved oxygen of 7.0 mg L^{-1}, total hardness of 340 mg L^{-1}, pH of 7.6, turbidity of 1.5 NTU, and total dissolved solid content of 660 mg L^{-1}. Fish were maintained at 25 ± 2 °C and exposed to a 16-h light/8-h dark photoperiod. The water in the tank was partially changed every day with aerated tap water. During the acclimatization, the fish were fed with commercial food from the Feilong aquarium fishery at a day-rate of 1-1.5% of fish body weight.

LC_{50} determination, sub-acute exposure of glyphosate, and sampling
A total of 160 healthy fish were used in the acute toxicity bioassays to determine the 96 h LC_{50} of glyphosate. The toxicity test design and exposure concentrations were based on the Spearman-Kärber

method (Kärber, 1931) with modification (Zhang and Liu, 1997). Preliminary acute toxicity tests were conducted to determine the concentration range that causes 0-10% and 90-100% mortality in fish. Then, the acute toxicity testing was performed with 96 h of glyphosate-exposure in a 30 L glass jar at seven concentrations (771.70, 714.52, 661.58, 612.55, 567.22, 525.18, and 486.26 mg/L) of glyphosate, and one control group was treated with aerated tap water (0 mg/L of glyphosate). A total of 80 fish were randomly divided into 8 groups (7 treatment groups and one control group with 10 fish in each group), and they were exposed to glyphosate solution under semi-static conditions for 96 h. During the treatment, no food was provided, but saturated oxygen was maintained in the solution for every group, and the water for all groups was completely changed every day. Each test was performed in duplicate. During the period of testing, the fish behavior was observed and dead fish were counted and removed from the aquarium. After 96 h of glyphosate-exposure, the LC_{50} was calculated with SPSS13.0 software according to the recorded number of dead fish in every group.

For the sub-acute exposure of glyphosate, 54 fish with the same body weight as above were randomly divided into three groups; two groups were the glyphosate-treatment groups (1/10 of 96 h LC_{50} and 1/5 of 96 h LC_{50}) and one was the control with aerated tap water. There were 18 fish in each group. The fish in the treatment group were exposed to glyphosate solution at concentrations of 1/10 of 96 h LC_{50} or 1/5 of 96 h LC_{50} under semi-static conditions for 168 h. During the treatment, no food was provided to avoid interference or an adverse effect on the following biochemical assay from differences in ingestion and digestion between the treatment and control groups, but saturated oxygen was maintained for the fish. The water in the three groups was completely changed daily. No fish died during the testing period. Each test was performed in duplicate. After 24, 72, and 168 h of glyphosate-exposure, respectively, 6 fish from every group were taken each time. The carp were anaesthetized with 100 mg/L MS-222 (Tricaine) and dissected; then, the tissues (liver, kidneys, and spleen) were rapidly isolated and washed with cold PBS. One part of the tissue was stored at -80 °C until it was biochemically assayed and the other was placed in the solution of 10% neutral-buffered formalin for histopathological analysis.

Assays of IFN-γ, IL-1β, and TNF-α

The IFN-γ, IL-1β, and TNF-α levels in the fish liver, kidneys, and spleen were determined using the kits from the Shanghai BOYAO Biotechnology, China, according to the manufacturer's instructions.

Histopathological examination

Sections of the liver, kidneys, and spleen were fixed in 10% neutral-buffered formalin for 24 h and were then dehydrated and embedded in paraffin. These sections were cut at a 5-6 μm thickness and stained with hematoxylin and eosin (H & E) before examination under a light microscope.

Statistical analyses

Data were analyzed using a one-way analysis of variance (Tukey) followed by a least significant difference determination with SPSS 13.0 for Windows. A P value less than 0.05 was considered statistically significant.

Results

The LC_{50} of glyphosate for common carp

The results of the acute toxicity test showed that the 96 h LC_{50} value of glyphosate for common carp was 520.77 mg L^{-1} with 95% confidence limits of 481.55-548.73 mg L^{-1}. Additionally, the fish behavior in the treatment group was different from that in the control group; for example, the treated fish became agitated. Also, they had an increased respiration rate and elevated opercular beat rate.

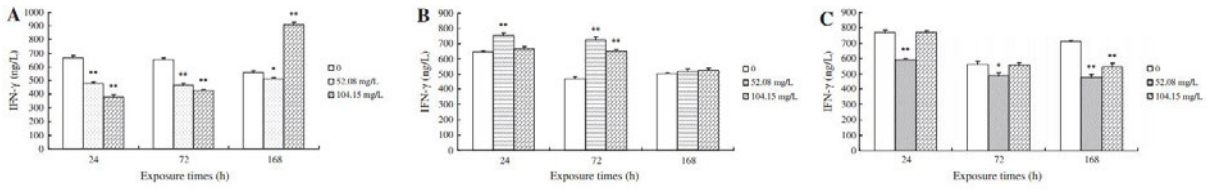
They swam quickly or jumped at the early periods of exposure, while they gradually became dull and weak and swam feebly and slowly, showing obvious symptoms of toxicity. At the late stage of testing, the treated fish vomited white foam and swam in a wandering pattern; finally, they sank to the bottom of the jar and died.

IFN-γ in the liver, kidneys, and spleen of common carp after subacute exposure of glyphosate for 168 h

The IFN-γ levels in the liver and spleen following glyphosate treatment were lower than the control

group throughout the period of sub-acute testing (except for an increase in the 104.15 mg/L group at the end of testing) (Fig. 1A and C). However, the IFN-γ level in the kidneys of the treatment groups was generally increased compared to the control groups (Fig. 1B).

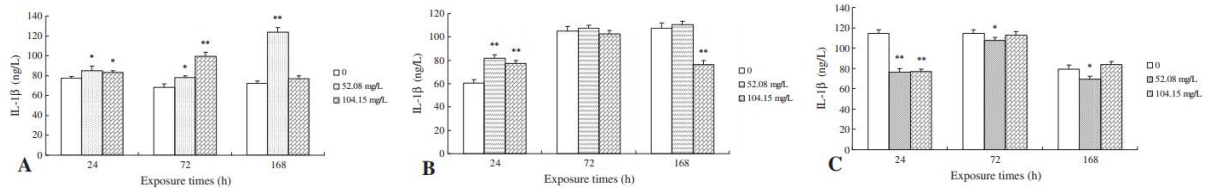
Figure 1. IFN-γ levels in the liver, kidneys, and spleen of common carp exposed to 52.08 or 104.15 mg/L of glyphosate for 168 h. The experiment was performed in duplicate and data are shown as the mean ±SD. Asterisks denote a response that is significantly different from the control group (*p < 0.05, **p < 0.01). (A) Levels of IFN-γ in the liver, (B) kidneys and (C) spleen.



IL-1β levels

The IL-1β levels in the fish liver, kidneys, and spleen are demonstrated in Fig. 2; the liver IL-1β level in the treatment fish was almost completely increased (Fig. 2A), while it decreased in the spleen (Fig. 2C). For the fish kidneys, changes in the IL-1β levels had a pattern that was different from that of the liver or spleen; first, there was an increase at 24 h, which was invariant at 48 h and then decreased at the end of testing (Fig. 2B).

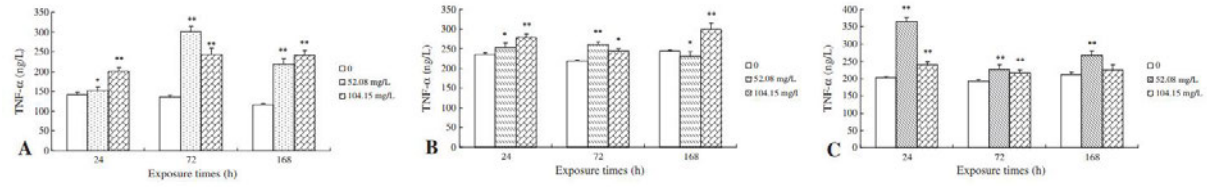
Figure 2. IL-1β levels in the liver, kidneys, and spleen of common carp exposed to 52.08 or 104.15 mg/L of glyphosate for 168 h. The experiment was performed in duplicate and data are shown as the mean ±SD. Asterisks denote a response that is significantly different from the control group (*p < 0.05, **p < 0.01). (A) Levels of IFN-γ in the liver, (B) kidneys and (C) spleen.



TNF-α level

On the whole, the TNF-α levels in the tissues of the glyphosate treated fish were increased compared to the control fish with the exception of the decrease in the kidneys (52.08 mg/L at 168 h) (Fig. 3).

Figure 3. TNF-α levels in the liver, kidneys, and spleen of common carp exposed to 52.08 or 104.15 mg/L of glyphosate for 168 h. The experiment was performed in duplicate and data are shown as the mean ±SD. Asterisks denote a response that is significantly different from the control group (*p < 0.05, **p < 0.01). (A) Levels of IFN-γ in the liver, (B) kidneys and (C) spleen.



Histological examination

Remarkable damage, such as intracytoplasmic vacuolization, nucleus distortion and pyknosis, and cellular swelling were found in the livers of the treated fish. Moreover, there was vacuolization of the renal parenchyma and intumescence of the renal tubule in the kidneys from the glyphosate-exposed fish. In addition, visible damages, such as vacuolization, focal necrosis, cells with double nuclei, and nuclear

pyknosis, were also found in the spleens of glyphosate-treated fish.

Conclusion

In the present study, the results of histopathological observation indicate that glyphosate causes obvious injury to the liver, kidneys, and spleen of the fish in the higher concentration group (104.15 mg/L glyphosate), including cellular swelling and intracytoplasmic vacuolization within the liver and spleen as well as vacuolization of the renal parenchyma and intumescence of the renal tubule in the kidneys.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Endpoints presented are not relatable to an EU level ecotoxicological reguatory risk assessment (enzyme, cellular and molecular level endpoints). In addition, the test substance used was a commercial formulation identified as a 50% soluble powder. This is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276) and therefore the article is not relevant for the renewal. Moreover, the fish sizes in the acute test were more than 8 times greater than the maximum permitted weight for fish acute testing for regulatory purposes. The presented results should therefore be treated with caution.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The acute toxicity of glyphosate on common carp was first determined; then, the contents of interferon- γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) and histopathological alterations in the liver, kidneys, and spleen of common carp exposed to 52.08 or 104.15 mg/L of glyphosate for 168 h were also determined and evaluated.

- Glyphosate has low toxicity on common carp (96 h LC50 = 520.77 mg/L).
- Glyphosate-exposure alters the contents of cytokines.
- Glyphosate caused histopathological damage to common carp.
- Glyphosate has immunotoxic effects on common carp.

Sublethal effects were investigated at concentrations of 52.08 and 104.15 mg/L of glyphosate (for 168 h). These concentrations are far from those expected under realistic conditions of use.
The formulation tested contains 50% glyphosate and is a soluble powder. The representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable.
Total hardness was of 340 mg/L, no analytical verification is available, so potential interaction with ions in the environmental conditions of this study are unknown. In OECD 203, the recommended hardness for common carp is 40 to 250 mg/L (preferably less than <180). Fish were rather big, RMS doubts their sensitivity. Overall, despite statistically significant, the effects do not seem concentration related. The results presented graphically only.
The results of histopathological observation indicate that glyphosate causes injury to the liver, kidneys, and spleen of the fish at a concentration of 104.15 mg L-1 glyphosate, i.e. at a concentration exceeding the expected exposure concentration for the intended uses.
The study is less relevant but supplementary (due to the uncertainty around the test item) and not reliable enough for risk assessment purpose.

Data point:	CA 9
Report author	Rocha T. L. *et al.*
Report year	2015
Report title	Proteomic and histopathological response in the gills of *Poecilia reticulata* exposed to glyphosate-based herbicide.
Document No	Environmental toxicology and pharmacology (2015), Vol. 40, No. 1, pp. 175-86
Guidelines followed in study	OECD (1992).
Deviations from current test guideline	Not mentioned
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Cellular and molecular level endpoints are discussed that are not relevant to EU level ecotoxicological regulatory risk assessment. Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). Observed findings relate to a formulation that is not the representative formulation for the glyphosate EU renewal. Thus the article is not relevant for the renewal.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Roundup Transorb was used.
The study authors indicated the the tested formulation was more toxic to fish than pure glyphosate and that higher toxicity was linked to the toxic effects of surfactant POEA or synergistic effect between the different components of the formulation.

In this study, it is clearly stated by the study authors that the tested formulation included the surfactant polyethoxylated tallow amine (POEA). Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Therefore it is not possible to discriminate between glyphosate and POEA.

The study is considered not relevant by RMS.

Data point:	CA 9
Report author	Silveira T. *et al.*
Report year	2019
Report title	Roundup® Herbicide Decreases Quality Parameters of Spermatozoa of Silversides *Odontesthes Humensis*
Document No	Bulletin of Environmental Contamination and Toxicology (2019), Vol. 102, No. 1
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The silverside (*Odontesthes humensis*) is a very interesting model for toxicological studies due to its high sensitivity and need for good water quality. The aim of this study was to evaluate the effects of Roundup on spermatozoa of *O. humensis*, after acute exposure. The fish were exposed to 0 and 7.8 mg L^{-1} (a.e.) of glyphosate, respectively. Through computer-assisted sperm analysis, a significant decrease in concentration, total and progressive motility, average path distance, straight line distance, path average velocity, curved line velocity, straight line velocity linearity, wobble, amplitude of lateral head displacement, cross beat frequency, and motility period of silverside spermatozoa exposed to Roundup was observed. Also, increase in membrane fluidity, ROS production and lipid peroxidation and a decrease in the mitochondrial functionality was observed in spermatozoa of Roundup exposed silversides. It was demonstrated that Roundup exposure in a concentration that can be achieved in natural water bodies soon after its application in fields is able to cause losses in several sperm quality parameters, consequently decreasing the fertilization potential of *O. humensis* spermatozoa.

Materials and methods

The silversides *Odontesthes humensis* used in this study came from eggs collected from wild broodstock in Lagoa Mirim and hatched in tanks. The fish were 1.5 years old and had mean length of 15.2 ± 2.2 cm and mean mass of 25.7 ± 11.2 g at experimental time. The silversides were sustained in 1000 L cylindrical plastic tanks, being fed three times a day with commercial feed (Supra, 38% crude protein) until satiety. The sides of tanks were opaque to reduce visual stress. The animals were kept since hatching in environmental water collected from Arroio Chasqueiro, less than 30 km from the collection site. The water parameters were pH 7.0 ± 0.7, temperature 13.3 ± 1.8°C, dissolved oxygen 9.7 ± 0.6 mg L^{-1}, total ammonia levels 0.4 ± 0.2 mg L^{-1}, and salinity 3.25 ± 0.67 ppt since the beginning of acclimation until the end of experimental period. The acclimation period was four weeks and experimental period was 24 h. No fish mortality was observed in any of the experimental groups of this study. Also, no difference between length ($p = 0.93$) or mass ($p = 0.90$) of silversides of both control and exposed groups were detected. The experimental setup consisted in a control and a Roundup treatment both in duplicate totaling in four tanks with five fish each.

The control group was exposed to the water in absence of Roundup herbicide. The Roundup group was submitted to acute exposure of Roundup herbicide on the nominal concentration of 10 mg L^{-1} (a.e.) of glyphosate for 24 h. After the exposure, the fish were anesthetized with 50 mg L^{-1} of benzocaine and euthanized by decapitation. The testes were dissected and stored in the extender Beltsville Thawing Solution (Minitüb, Germany) at 4°C for 1 h until the sperm analysis. The real concentration of glyphosate was measured in water samples and a Shimadzu 2010 Plus equipped with a flame photometric detector (FPD) and a DB-17 fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) (J&W, USA) were used to perform the analyses.
The optimized column oven temperature program was from 125 to 200°C at 15°C min^{-1}. The total run time was 5 min. Helium was used as the carrier gas, maintained at a constant flowrate of 1.5 mL min^{-1}. A calibration curve (0.15–10 mg L^{-1} glyphosate) was built and used for glyphosate concentration determination in water samples. All injections were performed with 1 μL in the split flow (10:1) mode at 260°C. The limit of detection and quantification of the method was 0.05 mg L^{-1} and 0.15 mg L^{-1}, respectively. Recoveries for spiked matrices were higher than 80%. The method showed adequate linearity for the calibration curve ($r^2 = 0.999$). Measured concentrations of glyphosate in the media after the experimental period was 7.8 ± 1.46 mg L^{-1} (a.i.) of glyphosate (n = 6). Spermatozoa kinetic parameters were determined using-assisted sperm analysis (CASA) system (AndroVision 3.5, Minitub, Germany) combined with an Axio Scope. A1® optical microscope (Zeiss, Germany). For evaluations, five activations per sample were prepared and one to two fields per activation were analyzed up to 10 s. Each sample was investigated to include at least 500 cells. To perform the flow-cytometry analysis, the Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems, USA) was used. Each sample was exposed to 2 mM of Hoechst 33342–H33342 (Sigma-Aldrich, USA) for 5 min before each analysis. Events were detected by fluorochrome with violet laser (UV 405 nm) and photomultiplier (PMT) VL1 (450/40 nm) filter. The acquisition rate was 200 events s^{-1} totaling 20,000 events per sample. All assays were performed in duplicate. The non-sperm events were eliminated from analysis by scatter plots of FSC × SSC and negative fluorescence of H33342 events (debris). Plasma membrane integrity of spermatozoa were evaluated using LIVE/DEAD Sperm Viability Kit (Invitrogen, USA) (SYBR-14/ propidium iodide (PI) dye). Sperm plasma membrane fluidity was evaluated using two fluorescent probes to allow differentiation between low and high fluidity cells, merocyanine 540 (M540) and YO-PRO-1. The mitochondrial functionality of spermatozoa was evaluated using rhodamine 123 (Rh123) (Sigma-Aldrich, USA) and PI probes. The chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) probe (Invitrogen, Spain), a derivative of fluorescein, was used for detection of reactive oxygen species (ROS). This fluorescence probe was combined with PI (Sigma-Aldrich, USA). Only intact cells (PI negative) were selected and classified as spermatozoa. ROS production was obtained by the mean of the green fluorescence intensity. In order to evaluate plasma membrane lipid peroxidation (LPO), the sperm were loaded with 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY), a lipophilic membrane probe that changes irreversibly its fluorescence from red to green upon oxidation.
The normality of all results was tested using Shapiro-Wilk test. The unpaired t-test was employed to compare the length and the weight across the treated groups, since only these parameters presented normal distribution. In all other parameters the significant differences among medians were evaluated by the nonparametric Mann-Whitney test. Quantitative data were expressed as mean ± standard error of the mean (SEM).

Results

This study evaluated the effects of water contaminated with Roundup herbicide in spermatozoa of a wild silverside species. The present study also provided sperm characteristics for *O. humensis*. The knowledge of spermatozoa toxicology is important not only for an individual reproductive sphere but also for biomonitoring, since these cells, instead of whole animals, can be used as an alternative to detect environmental contaminants. Furthermore, the detection of negative impact of contaminants in reproductive cells may signify a more profound impact in future generations.
In the present study, the exposure to Roundup at concentration of 7.8 mg L^{-1} (a.e.) of glyphosate results in a significant decrease of the majority of kinetics parameters of the analyzed spermatozoa, which only

distance curved line (DCL) and straightness (STR) was not affected. In zebrafish (*D. rerio*), the exposure to 5 and 10 mg L^{-1} of pure glyphosate for 24 and 96 h also reduced the motility period and sperm motility but did not alter sperm cells concentration. In Rio de La Plata one-sided livebearer (*Jenynsia multidentata*), the treatment with the same Roundup formulation tested in this study at concentration of 0.5 mg L^{-1} (a.e.) of glyphosate for 24 h led to a significant reduction in cell concentration and motility percentage of spermatozoa from these fish. Membrane integrity was the only, among the non-kinetics parameters, that did not present a negative response after exposure to Roundup in silversides. Additionally, the exposure of up to 16 mg L^{-1} (a.e.) of Roundup did not affect the membrane integrity of oyster spermatozoa. Due to being the first protective barrier of the spermatozoa, its cell membrane has already been reported as more sensitive to different types of pollutants. However, herbicides can also produce negative effects in internal sperm cell functionalities without leading to loss of membrane integrity (Akcha et al. 2012). This corroborates to the idea that sperm toxicity on different parameters appears to be species-specific.

The present study reveals that membrane fluidity of silverside spermatozoa was higher in the fish exposed to Roundup. Besides this, there are no more studies in scientific literature that evaluated the relation between Roundup and membrane fluidity in sperm cells. However, the elevated level of polyethoxylated tallow amine (POEA) surfactant in Roundup formulation may act solubilizing the lipids from plasma membrane leading to an increase of membrane fluidity. This effect of surfactants has already been observed in other biological membranes (Pettitt and Buhr 1998; Glover et al. 1999). Mitochondrial function is one of the key parameters governing the fertilization (Akcha et al. 2012). Sperm cells with high mitochondrial function are more viable and probably increases the period of motility in fish, which results in higher rates of in vitro fertilization (He and Woods III 2004; Gillan et al. 2005). In *O. humensis*, the Roundup exposure causes a decrease in mitochondrial function.

The same pattern has already been observed in zebrafish exposed to 10 mg L^{-1} (a.e.) of glyphosate for 24 and 96 h (Lopes et al. 2014) and in guppy exposed to Roundup at 0.7 mg L^{-1} (a.e.) for 96 h (Harayashiki et al. 2013). The reduced mitochondrial respiration may lead to a decrease in ATP production and, consequently, to a lower metabolism and cell motility, observed in this study by CASA analysis (Christen et al. 1987; Perchec et al. 1995). The accumulation of ROS in sperm cells can result in cell damage and general loss of sperm quality, including damage of membrane structure; alterations in the mitochondrial function; and loss of fertilization capacity (Shiva et al. 2011). The exposure to Roundup led to an increase in ROS production levels in silverside sperm cells, which can be directly related to changes in cell membrane, mitochondrial functionality and spermatozoa motility, as it was observed by this study. Increases in the ROS production has already been reported in zebrafish spermatozoa after cryopreservation (Hagedorn et al. 2012). Furthermore, ROS levels were increased following the exposure to Roundup in Rio de La Plata one sided livebearer (Sánchez et al. 2017) and in guppy (Harayashiki et al. 2013), however these evaluations were carried out in non-reproductive cells. This is the first study that evaluated ROS production directly in sperm cells of a fish species. LPO generated by accumulation of ROS can cause a loss of membrane fluidity and integrity, affecting also the motility of spermatozoa, all of these characteristics are important for gamete fusion during the fertilization process (Hagedorn et al. 2012; Liu et al. 2015). Furthermore, the mitochondria also can be negatively affected by the increase of LPO due to its abundance of polyunsaturated fatty acids and poor in antioxidants (Agarwal et al. 2008).

In this study, the spermatozoa of silversides presented a significant decrease in motility and mitochondrial function and these phenomena can be directly related to the increase in LPO. However, the effect in the membrane fluidity may be masked by the action of the surfactant present in Roundup formulation. The increasing in LPO followed by Roundup exposure was observed in the Rio de La Plata one-sided livebearer liver (Sánchez et al. 2017) and European eel (*Anguilla anguilla*) erythrocytes (Guilherme et al. 2010), but none of these studies evaluated the effect of this herbicide directly in the fish sperm cells. The indiscriminate use of Roundup brings deleterious effects to fishes exposed to the contaminated environment, as well as to their future generations. Furthermore, Roundup effects before fertilization, such as male reproductive deficiency, also can be noticed.

Conclusion

No specific conclusion was provided by the authors. In the results and discussion, the authors summarized that a short-term Roundup exposure to realistic concentration present in natural water bodies following its application in fields is able to cause significant losses in several sperm quality parameters, decreasing consequently the potential of fertilization of *O. humensis* spermatozoa. The results generate great concern and raises a critical issue about the maintenance of future population of *O. humensis*.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Observations / endpoints based on effects on spermatazoa are not used in the EU ecotoxicological risk assessment, thus the findings cannot be related the EU renewal of glyphosate. In addition, it is not possible to confirm the identity of the test substance used in the study as only Roundup was mentioned. Roundup is a brand that contains multiple glyphosate-based herbicides, that contain different co-formulants. Of most importance to the toxicity profile associated with a particular product is whether that product tested contains polyoxyethyleneamine (POEA) as POEA surfactant is not permitted for use in formulated herbicidal products in the EU. However, Roundup Original (containing POEA) and POEA surfactant were discussed on multiple occasions in the paper. The surfactant system in the formulated product used in this study was not confirmed by the authors. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The effect of of Roundup on spermatozoa of the fish silverside (*Odontesthes humensis*) was investigated after acute exposure at concentrations of 7.8 mg L−1 (a.e.) of glyphosate (in Roundup formulation).
Effects were:

- a significant decrease in concentration, total and progressive motility, average path distance, straight line distance, path average velocity, curved line velocity, straight line velocity linearity, wobble, amplitude of lateral head displacement, cross beat frequency, and motility period of silverside spermatozoa
- increase in membrane fluidity, ROS production and lipid peroxidation
- decrease in the mitochondrial functionality

This study suggests that Roundup is able to cause losses in several sperm quality parameters, consequently decreasing the fertilization potential of O. humensis spermatozoa.

RMS considers these parameters relevant for the risk assessment. However the concentrations tested in laboratory conditions was high (7.8 mg acide equivalent/L) and above those expected in environmentally realistic conditions, so the study is not considered relevant. No intermediate concentration was tested.
In view of the formulation description, the use of the results to assess the toxicity of glyphosate as formulated in MON52276 is questionable. In the present study, the authors hypothesized that "*the elevated level of polyethoxylated tallow amine (POEA) surfactant in Roundup formulation may act solubilizing the lipids from plasma membrane leading to an increase of membrane fluidity*". While presence of POEA in the tested formulation is not clearly stated, RMS assumes its presence very likely as part of the study authors discussion and considers the study not relevant.

The study is considered not relevant by RMS.

Data point:	CA 9
Report author	Zebral Y. D. *et al.*
Report year	2018
Report title	A glyphosate-based herbicide reduces fertility, embryonic upper thermal tolerance and alters embryonic diapause of the threatened annual fish *Austrolebias nigrofasciatus*.
Document No	Chemosphere (2018), Vol. 196, pp. 260-269
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant:	Not relevant by full text

See RMS analysis in RMS comment box	

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The study aimed to evaluate acute (96 h) effects of Roundup Transorb R® exposure (0.36 or 3.62 mg a. e./L) in reproduction, diapause pattern and embryonic upper thermal tolerance (EUTT) of *A. nigrofasciatus*. For such, the researchers evaluated the number and diameter of embryos produced by exposed fish. Also, recently fertilized embryos were exposed and its diapause pattern was evaluated. Following 15 post exposure days (PED), the researchers evaluated the number of somite pairs and following 30, 35 and 40 PED and the researchers evaluated the proportion of pigmented embryos (PPE). Finally, the critical thermal maximum (CTMax) of exposed embryos was assessed. Results demonstrated that couples exposed to 0.36 mg a. e./L Roundup Transorb R® roduced less but larger embryos. Similarly, embryos exposed to 3.62 mg a. e./L Roundup Transorb R® had a reduced PPE following 30 PED. Finally, embryos exposed to 0.32 mg a. e./L Roundup had a CTMax reduction of 2.6 °C and were more sensitive to minor increases in heating rates.

Materials and methods

Animal collection and rearing
A total of 12 *A. nigrofasciatus* mating pairs were collected in a wetland located in the floodplains of the Padre Doutor stream (Patos-Mirim lagoon system, Rio Grande do Sul state, southern Brazil) and transferred to the aquatic laboratory located at UFPel (Universidade Federal de Pelotas), where the experiments were performed. Mating pairs were reared in 20 L aquaria. An artificial spawning nest containing peat moss was added to each aquaria and animals reproduced freely. Animals were acclimated to laboratory conditions for 3 weeks. During this period, fertilized embryos were manually collected once a week and reared according to literature. Following embryo collection or adult acclimation period, animals were distributed among treatment groups as described in experimental design section. During acclimation period and during the different experimental periods, animals were maintained under constant aeration and controlled temperature (21 °C) and photoperiod (12 h:12 h), according to previously described methods. Water temperature, pH and NH_3 (Nutrafin NH_3/NH_4 test, Hagen, Mansfield, MA, USA) were measured once a week. Embryonic development was determined following Wourms (1972) classification.

Roundup Transorb R® exposure
Roundup exposure proceeded similarly to our previous work (Zebral et al., 2017). The treatment groups consisted of one or two nominal concentrations of the herbicide, one low and environmentally-relevant concentration (0.36 mg a. e./L) and an elevated concentration (3.62 mg a. e./L) and a control group (no herbicide addition). Depending on the experiment, only one or both exposure concentrations were used. The commercial formulation used was Roundup Transorb R® (Brazil, MAPA SOB Nº 09306, 480 g/L glyphosate acid equivalents) and concentrations are expressed as glyphosate acid equivalents (a.e). For the reproduction experiment, exposure solutions were prepared by direct application of the commercial formulation to experimental units. In the case of the other experiments, exposure solutions were firstly prepared by direct application of the commercial formulation in a glass aquarium filled with clean water (5 L volume), then the contaminated water was added to experimental units. Following extensive mixture of the experimental media, the animals were added to the respective experimental unit. The experiments were conducted for 96 h, without water renewal. Also, the exposure period used in the work is much smaller than the glyphosate half-life reported by literature. Glyphosate concentration was measured in water samples. Briefly, extraction was performed by the liquid-liquid method using 150 ml of sample (adjusted pH: 2) with a mix of ethyl acetate and acetonitrile (1:1; 3 x 20 ml). This mixture was added with NaCl (4 g) to facilitate glyphosate organic phase partitioning and agitated for 3 min.

The aqueous layer formed was discarded and the organic phase was evaporated to dryness with nitrogen. The derivatization reaction was performed by adding 800 ml of trifluoroacetic anhydride (TFAA) and 400 ml of trifluoroethanol (TFE). Samples were held for 1 h at 100 °C and evaporated to dryness with nitrogen. Finally, samples were re-suspended in 1 ml of ethyl acetate and analyzed by gas chromatography with flame photometric detection.

Experimental design

Reproduction experiment
Following acclimation period, adult *A. nigrofasciatus* were exposed to Roundup Transorb R. Animals were distributed among experimental units at random. Each experimental unit consisted of 7 L aquaria with a *A. nigrofasciatus* mating pair separated by a transparent and holed acrylic divisor, in order to prevent aggression but permit visual and chemical sexual stimulation. The experiment was conducted with an exposure group (0.36 mg a. e./L) and a control group (no herbicide addition) for 96 h. The experiment was conducted with 4 aquariums per group, and each aquarium had one mating pair (n = 4). Mortality was daily evaluated. Following exposure period, the experimental media was changed and the experimental units were filled with clean water. Subsequently, an artificial spawning nest containing peat moss was added to each aquarium and the acrylic divisor was removed. Finally, *A. nigrofasciatus* mating pairs were free to reproduce. Following 24 h, the spawning nest was removed and the produced embryos were collected, counted and had its diameter measured (20 embryos per treatment). For this, embryos were transferred to a depression slide and were analyzed under a light microscope with an ocular micrometer (model 1718, Bioval, Shanghai, China). The number of produced embryos is expressed per gram of female wet weight (embryos/g). Animals were not fed during the experiment.

Pattern of embryonic diapause experiment
In order to evaluate long term effects of an acute exposure to Roundup Transorb R, embryos acquired following at least one week of acclimation period were exposed to the herbicide. The experiment consisted of two exposure groups (0.36 and 3.62 mg a. e./L) and a control group (no herbicide addition). Each group consisted of 40 embryos divided at random in four containers. All the embryos were at the dispersion fase (Wourms, 1972). Animals were exposed for 96 h in 60 ml containers. Following exposure period, embryos survival was checked and the animals were transferred to 60 ml containers filled with clean water. All the embryos were analyzed in four post exposure periods and different endpoints were evaluated. Following assessments, embryos were returned to the respective container. Following 15 post exposure days (PED), the researchers evaluated the number of somite pairs. In this case, the number of somite pairs of each individual embryo was considered as an independent observation (n = 40). Similarly, following 30, 35 and 40 PED the researchers evaluated the proportion of pigmented embryos. In this case, the value acquired for each experimental unit was considered as an independent observation (n = 4). The researchers chose to evaluate these endpoints in order to access possible alterations in the embryonic developmental rate that would indicate alterations in the diapausing pattern. In order to access these endpoints, embryos were transferred to a depression slide and analyzed under a light microscope. Mortality was daily evaluated.

Embryonic thermal tolerance experiment
In order to evaluate the effect of Roundup Transorb R exposure in the upper thermal tolerance of *A. nigrofasciatus* embryos, eggs acquired following at least one week of acclimation period were exposed to the herbicide and the CTMax was accessed. The experiment was performed with one exposure group (0.36 mg a. e./L) and a control group (no herbicide addition). Each group consisted of 10 embryos in the developmental stage 43, described by Wourms (1972). Animals were exposed to the herbicide for 96 h in 60 ml containers. Following exposure period, embryos survival was checked and the CTMax was assessed. For this, embryos were individually transferred to a petri dish placed in a stereomicroscope containing 14 ml of clean water (no herbicide addition) at 21 °C. The temperature of the water present in the petri dish was elevated by the heat produced by the stereomicroscope lamp. The water temperature was assessed by a digital thermometer (0.01 °C) and annotated every 1 min. The embryos were constantly observed in the stereomicroscope and the experiment was finalized when animals displayed absence of heart beat for 5 s (endpoint). When reaching at the endpoint, the temperature was annotated as the animal's CTMax and the embryo was transferred to clean water at 21 °C. The CTMax was validated by embryo survival in the following 96 h. In order to produce homogeneous heating rates, the temperature of the stereomicroscope lamp was also assessed and maintained constant (56 °C). The embryos were exposed to the mean heating rate of 1.3 ± 0.06 °C/min. With the intention to minimize methodological bias, all CTMax evaluations were performed blind folded. Therefore, the observer could not see the water temperature that embryos were experiencing neither known if the animal was from exposition or control group.

Statistical analysis
Data are expressed as mean ± standard error. Differences among treatment groups were assessed using analysis of variance (one-way ANOVA or Student's t-test). Parametric assumptions were assessed. Residuals were evaluated to test if the experimental data had a normal distribution and if the variances were homogeneous using the Kolmogorov-Smirnov and Levene's tests, respectively. The independency of observations was also assessed by the Durbin-Watson test. If parametric assumptions were not met, data were mathematically (exponential) transformed and assumptions were tested again. Experimental groups differences were assessed by the Tukey's test. The relationship among embryos CTMax and mean heating rates was assessed by regression analysis. In all cases, the significance level adopted was 95% ($\alpha = 0.05$). All statistical analyses were performed using the SigmaPlot 12.0 software (Systat, San Jose, CA, USA).

Results

Water parameters
For the reproduction experiment, mean water temperature and pH were 20.75 ± 2.17 °C (control); 20.5 ± 2.01 °C (0.36 mg a. e./L) and 7.52 ± 0.06 (control); 7.48 ± 0.1 (0.36 mg a. e./L), respectively. Likewise, the mean water temperature and pH for the embryonic development experiment was 20.4 ± 0.2 °C (control); 20.5 ± 0.3 °C (0.36 mg a. e./L); 20.4 ± 0.4 °C (3.62 mg a. e./L) and 7.4 ± 0.07 (control); 7.4 ± 0.1 (0.36 mg a. e./L); 7.4 ± 0.1 (3.62 mg a. e./L), respectively. Similarly, the mean water temperature and pH for the thermal tolerance experiments were 20.6 ± 0.1 °C (control); 20.5 ± 0.2 °C (0.36 mg a. e./L) and 7.5 ± 0.1 (control); 7.4 ± 0.2 (0.36 mg a. e./L), respectively. NH_3 concentration in the experimental media was always < 0.25 mg/L.
At the beginning of the experiment (immediately after water contamination with Roundup Transorb R), measured glyphosate concentrations were similar to target nominal concentrations in all experiments. Mean daily decay of glyphosate concentration was 15.6 ± 4.4% for the reproduction experiment 0.36 mg a. e./L). For the experiments with embryos, glyphosate mean daily decay was 15.3 ± 6.7% (0.36 mg a. e./L) and 6.0 ± 7.2% (3.62 mg a. e./L). Glyphosate concentration in the control groups were always < 0.002 mg a. e./L.

Reproduction
The number of embryos and the embryos diameter were evaluated following Roundup Transorb R exposure for 96 h to evaluate herbicide effects on reproduction. Compared to control conditions,

Roundup Transorb R exposure produced significant alterations in both parameters evaluated. Exposure to 0.36 mg a. e./L of the herbicide reduced the number of fertilized embryos/g produced by *A. nigrofasciatus* couples ($F_{1,6} = 5.64$; $p < 0.05$) (Fig. 1A) and slightly elevated embryos diameter ($F_{1,52} = 6.71$; $p < 0.05$) (Fig. 1B). No mortality was observed. Also, mating pairs biometric information (weight and length) are expressed in Table 1. Considering each sex, no differences were observed among experimental groups.

Figure 1. A) Number of embryos per gram of female produced in 24 h by *A. nigrafasciatus* couples maintained in control condition (no herbicide addition in the water) or exposed for 96 h to 0.36 mg a.e./L Roundup Transorb R®. Data are expressed as mean ± standard error. Asterisk indicate significant different mean values among experimental groups (t-test; n = 4; $p < 0.05$). B) Embryonic diameter produced by *A. nigrofasciatus* couples maintained in control condition (no herbicide addition in the water) or exposed for 96 h to 0.36 mg a. e./L Roundup Transorb R®. Data are expressed as mean ± standard error. Asterisk indicate significant different mean values among experimental groups (t-test; n = 20; $p < 0.05$).

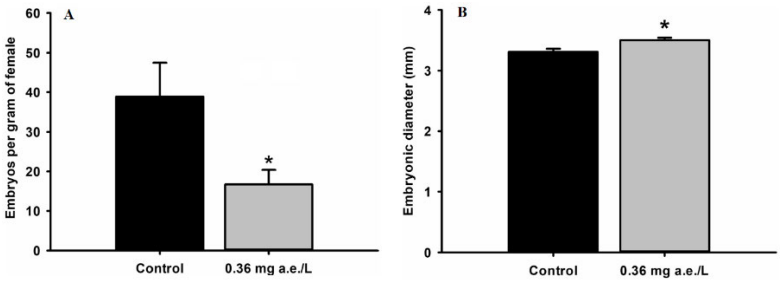


Table 2. Weight (g) and total length (cm) of annual fish *A. nigrofasciatus* acutely (96 h) exposed to 0.36 mg a.e./L Roundup Transorb R® and control group (no herbicide addition in the water). Considering each sex, no significant differences were observed among experimental groups (one-way ANOVA; n = 4; $p > 0.05$).

Treatment	Sex	Weight (g)	Total length (cm)
Control	Male	0.82 ± 0.06	4.02 ± 0.10
	Female	0.57 ± 0.03	3.47 ± 0.09
0.36 mg a.e./L	Male	0.96 ± 0.17	4.20 ± 0.28
	Female	0.68 ± 0.06	3.72 ± 0.07

Pattern of embryonic diapause

In order to assess if an acute exposure (96 h) to two Roundup Transorb R concentrations (0.36 and 3.62 mg a. e./L) could produce long term effects in the embryonic diapause of *A. nigrofasciatus*, the researchers evaluated the number of somite pairs following 15 PED and the percentage of pigmented embryos following 30, 35 and 40 PED. These results are presented in Table 2. Significant effects of herbicide exposure were only seen in the proportion of pigmented embryos following 30 PED, considering that Roundup Transorb R exposure to 3.62 mg a. e./L reduced this parameter in comparison to control condition ($F_{2,9} = 4.50$; $p < 0.05$) (Fig. 2). Also, non-significant reductions in the proportion of pigmented embryos were seen following 30 PED to 0.36 mg a. e./ L Roundup Transorb and following 35 PED to 3.62 mg a. e./L Roundup Transorb (Table 2). No mortality was observed.

Figure 2. Percentage of *A. nigrofasciatus* pigmented embryos after 30 days of acute (96 h) exposition to 0.36 or 3.62 mg a. e./L Roundup Transorb R® and control group (no herbicide addition in the water). Data are expressed as mean ± standard error. Different letters indicate significant different mean values among experimental groups (one-way ANOVA; n = 4; $p < 0.05$).

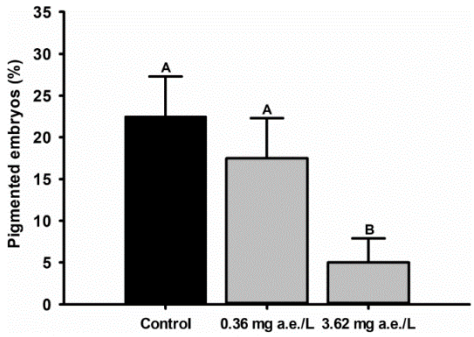


Table 2. Endpoints evaluated in annual fish *A. nigrofasciatus* embryos after 15, 30, 35 or 40 days of acute (96 h) exposition to 0.36 or 3.62 mg a.e./L Roundup Transorb R® and control group (no herbicide addition in the water). Data are expressed as mean ± standard error. Different letters indicate significant different mean values among experimental groups (one-way ANOVA; n = 40 for somite pairs and n = 4 for percentage of pigmented embryos; $p < 0.05$).

Endpoints evaluated	Post Exposure Days	Control	0.36 mg a.e./L	3.62 mg a.e./L
Somite pairs	15	13.72 ± 0.94^{A}	13.95 ± 1.09^{A}	14.17 ± 1.19^{A}
Pigmented embryos (%)	30	22.50 ± 7.80^{A}	17.50 ± 4.80^{A}	5.00 ± 2.80^{B}
Pigmented embryos (%)	35	32.50 ± 7.50^{A}	22.50 ± 8.55^{A}	15.25 ± 6.40^{A}
Pigmented embryos (%)	40	42.50 ± 7.50^{A}	37.50 ± 7.50^{A}	35.00 ± 6.45^{A}

Embryonic thermal tolerance

The CTMax of embryos was evaluated in order to assess effects of Roundup Transorb R on thermal tolerance. Exposure to 0.36 mg a. e./L of the herbicide significantly reduced embryos CTMax from 38.7 ± 1.69 °C (control) to 36.12 ± 1.08 °C (0.36 mg a. e./L) ($F_{1,17}$ =15.11; $p < 0.001$). Additionally, regression analysis showed that the CTMax of exposed animals reduced with minimal elevations in mean heating rates ($y = 41.47\text{-}8.03x$; n = 9; $r^2 = 0.3$; $p = .1$). Inversely, the CTMax of control animals was less affected by variations in mean heating rates ($y = 40.24\text{-}2.39x$; n = 10; $r^2 = 0.008$; $p = 0.8$). The mean heating rates was not statistically different among control (1.28 ± 0.06 °C/min) and exposed animals (1.32 ± 0.07 °C/min). No mortality was observed.

Conclusion

The present work aimed to evaluate the effect of Roundup Transorb R® exposure in the reproduction, the embryonic diapause and the embryonic upper thermal tolerance of the annual fish *A. nigrofasciatus*. The researchers demonstrated that 96 h exposure to the low and environmentally relevant Roundup Transorb R® concentration of 0.36 mg a. e./L can reduce fish fertility and embryonic thermal tolerance. Also, the researchers demonstrated that embryos acutely exposed (96 h) to the more elevated Roundup Transorb R® concentration of 3.62 mg a. e./L had alterations in the diapausing patterns even after 30 days of the acute exposure period. When taken together, the results of the present study have a special relevance to the conservation of annual fish populations, considering that it was demonstrated that Roundup Transorb R® can alter key aspects that permit these animals to survive in extreme environments such as ephemeral wetlands. Therefore, the information presented in the present study may help to improve the conservation of fish populations, especially the annual fishes from southern Brazil.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The product tested in this study, (Roundup Transorb R®) is not the representative formulation for the glyphosate EU renewal. Thus, the article is not relevant for the renewal. In addition, the presented data are not considered relevant for use in regulatory risk assessment, as they are single rates and not derived end-points. Moreover, this study was conducted in Brazil and can be difficult to extrapolate to EU (local native species, geo-climatic properties, land-uses and agricultural practices differ from EU).

Further points of clarification:
Roundup Transorb R® commercialized in Brazil (Brazil, MAPA SOB Nº 09306) contains 480 g/L glyphosate acid and was formulated using glyphosate potassium salt whereas the representative formulation MON 52276 does have a nominal glyphosate acid equivalent (a.e.) content of 360 g/L and was formulated glyphosate isopropyl-ammonium salt.

Assessment and conclusion by RMS:

Roundup Transorb R®was used. Surfactants were not stated in this study.
It is very likely that the tested formulation included the surfactant polyethoxylated tallow amine (POEA) as in an other study Rocha T. L. et al., 2015, (also conducted in Brazil) "Transorb" was used. In this study it was clearly stated that Transorb contains POEA. Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Therefore it is not possible to discriminate between glyphosate and POEA.
The study is considered not relevant by RMS.

4. Aquatic invertebrates

Data point:	CA 8.2.4, CP 10.2.2
Report author	Deepananda Ashoka K. H. M. *et al*.
Report year	2011
Report title	Acute toxicity of a glyphosate herbicide, Roundup®, to two freshwater crustaceans.
Document Source	Journal of the National Science Foundation of Sri Lanka (2011), Vol. 39, No. 2, pp. 169-173
Guidelines followed in study	US EPA-821-R-02-012
Deviations from current test guideline	Minor deviations
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as provided by the applicant is reported here.

Full summary of the study according to OECD format

The acute toxicity of a glyphosate herbicide Roundup® contaminating freshwater ecosystems in Sri Lanka was investigated with two species of the most common freshwater crustaceans: calanoid copepod (*Phyllodiaptomus annae* Apstein) and decapod shrimp (*Caridina nilotica* P. Roux). LC_{50} values in adult males were determined using standard toxicity assays under static conditions. The 48h LC_{50} value for *P. annae* was estimated as 1.06 mg/L and the values of 72 h and 96 h LC_{50} for *C. nilotica* were 107.53 and 60.97 mg/L, respectively. The present study reveals that Roundup® may cause a significant impact on native non-target organisms. Further studies are needed to establish the toxic effect of Roundup® to the very important structural group in the aquatic ecosystems in Sri Lanka.

Materials and methods

Adult male calanoid copepod *Phyllodiaptomus annae* Apstein (total length 1.19 ± 0.02 mm, excluding caudal setae) and adult male decapod shrimp, *Caridina nilotica* P. Roux (total length 1.74 ± 0.23 cm and wet weight 0.047 ± 0.008 g) were selected for the acute toxicity bioassay. They were acclimatized to laboratory conditions for 48 h before being used in the bioassay. Roundup® (360 g/L, purity 98%) was purchased from a local retail outlet, and dosing solutions were prepared by diluting with water to give the nominal concentrations. Prior to the definitive test, preliminary screening with a wide concentration series was carried out to determine the definitive exposure ranges for testing the chemical. Tests were conducted in accordance with the EPA guidelines.

Bioassay with *P. annae* was conducted in 50 mL acid washed glass beakers filled with 40 mL of source water filtered through Whatman GF/C glass microfiber filters. Some characteristics of this water were: temperature, 28 ± 1ºC; dissolved oxygen, 8.4-9.2 mg/L; pH, 7.3-7.8; and conductivity, 200-215 mS. Groups of test organisms, each consisting of 20 individuals were selected at random and placed into test chambers indiscriminately arranged on a bench in the laboratory. Different concentrations of Roundup® (0.1, 0.2, 0.4, 0.8, and 1.6 mg/L) were added to the experimental beakers and mortality was assessed at the end of the test (48 h). Organisms with no response at all were considered dead. The toxicity test consisted of a control and 5 concentration groups with 6 replications per concentration group, resulting in a total of 120 individuals for each concentration. Test solutions were neither renewed nor aerated.

The bioassay with *C. nilotica* was performed in glass aquaria (23x23x35 cm) with a 4 L capacity. Test chambers were filled with 2 L of dechlorinated tap water having the following physicochemical characteristics: temperature, 27.5 ± 0.5ºC; pH, 6.8-7.3; dissolved oxygen, 8.3-8.9 mg/L; and conductivity, 167-229 mS. Groups of experimental animals, each consisting of 20 individuals, were selected at random and placed into aerated aquaria. The toxicity test consisted of a control and four concentration groups with four replications per group, resulting in a total of 80 individuals for each concentration. The different concentrations of Roundup® (20, 40, 80 and 160 mg/L) were added to the experimental aquaria. Mortality was assessed at 24, 48, 72 and 96 h after the start of the test. Test solutions were renewed every day, so the animals were transferred to new aquaria containing fresh medium every 24 h. Animals were not fed during the 48 h adaptation period and throughout the experiment. Controls were treated in the same way but without adding of pesticide to the test water.

All data were expressed as arithmetic means ± SD. Statistical analysis was performed using SPSS for Windows (Version 10.0.1). Mortality rates among exposure groups and controls were compared using the ANOVA least significant difference (LSD) test. Median lethal concentrations (LC_{50}) and their respective confidence intervals (95%) were calculated by means of the EPA probit analysis programme.

Results

The percentage mortality of copepods in each concentration group became progressively higher as the concentration of exposure increased, and the mean mortality in all of the five concentration groups exposed to Roundup® was significantly higher than that of the controls. Similarly, the percentage mortality in the shrimp exposed to glyphosate showed a progressive increase from the lowest to the highest concentration group. Mean mortality rates in the shrimp exposed to all concentrations at 72 and 96 hours were significantly higher than that of controls (Table 1). Control mortality was zero.

Table 1: Mean mortality of P. *annae* Apstein (a copepod) and C. *nilotica* P. Roux (a shrimp) exposed to different concentrations of glyphosate in Roundup®

	Percentage mortality (mean values)			
	24 h	48 h	72 h	96 h
P. annae (adult males)				
Control	-	0	-	-
Glyphosate exposed				
0.1 mg/L	-	1.33*	-	-
0.2 mg/L	-	1.67*	-	-
0.4 mg/L	-	2.67**	-	-
0.8 mg/L	-	4.00***	-	-
1.6 mg/L	-	19.00***	-	-
C. nilotica (adult males)				
Control	0	0	0	0
Glyphosate exposed				
20 mg/L	1.25	3.75	8.75*	8.75*
40 mg/L	3.75	8.75*	16.25***	16.25***
80 mg/L	8.75*	21.25***	38.75***	38.75***
160 mg/L	12.50***	30.00***	53.75***	53.75***

For a specific test species, mortality data are significantly different from the respective control group (*p<0.05, **p<0.01, ***p<0.001)

Within each group, the mean mortality rate became progressively higher as duration of exposure increased. A statistically higher mean mortality rate in animals exposed to the lowest concentration was not observed until the end of third day (72 hours). Similarly, in another concentration group (40 mg/L), a significantly higher mean mortality rate was not observed until the end of the second day (48 hours).

The calculated 48 hour acute LC_{50} value (95% confidence limits) of Roundup®, using static bioassay of adult male *P. annae* was 1.059 mg/L (0.985 - 1.134), and the calculated LC_{50} values at 72 and 96 hours for Roundup® in adult male *C. nilotica* were 107.53 and 60.97 mg/L, respectively (Table 2).

Table 2: Acute toxicity of glyphosate in Roundup® for adult males of the copepod, *P. annae* Apstein and the shrimp, *C. nilotica* P. Roux

Test species	LC50 (mg/L)	95% confidence limits	Slope ± SE	Intercept ± SE
P. annae				
48 h	1.059	0.985 - 1.134	8.96 ± 0.89	4.77 ± 0.14
C. nilotica				
72 h	107.53	88.04 - 140.49	1.88 ± 0.25	1.18 ± 0.46
96 h	60.96	53.21 - 70.04	2.71 ± 0.27	0.17 ± 0.48

Conclusion

Based on the ecotoxicological risk categories for aquatic organisms and derived 48 and 96 hours LC_{50} values, Roundup® appears to be moderately toxic to *P. annae* and slightly toxic to *C. nilotica*. The acute toxicity of Roundup® in the two species of crustaceans tested here was found to be much lower than the values available from standard ecotoxicology laboratory testing.
The mean mortality rates in tested *P. anne* and *C. nilotica* have revealed that even a very low dose of Roundup® (0.1 mg/L and 20 mg/L, respectively) can severely affect both species, which are fundamental links in the dynamics of the tropic chain in Sri Lankan water bodies.
C. nilotica is more sensitive to Roundup® than other freshwater shrimps already tested for glyphosate herbicide resistance. The results of the present study emphasize that toxicity testing on multiple species is often required, because different species have different tolerances to Roundup®

The calculated 48 hour acute LC_{50} value (95% confidence limits) of Roundup®, using static bioassay of adult male *P. annae* was 1.059 mg/L (0.985 - 1.134), and the calculated LC_{50} values at 72 and 96 hours for Roundup® in adult male *C. nilotica* were 107.53 and 60.97 mg/L, respectively.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: After exposure to Roundup® the 48 hour acute LC_{50} for adult copepod *Phyllodiaptomus annae* was determined to be 1.059 mg/L. This endpoint is questionable as there was only 19% mortality at the highest exposure concentration in the test (1.6 mg/L). For the second species, the 72 and 96 hour LC_{50} for decapod shrimp *Caridina nilotica* was determined to be 107.53 and 60.97 mg/L, respectively. However, the mean percenatge mortality at both timepoints was identical from Table 1 in the paper. As there are no biological data presented in the paper, the observed mortality and the LC_{50} calculation cannot be confirmed. The formulation content is identified as Roundup® (360 g/L, 98%). However, the presented purity appears to be incorrectly stated, as a formulation with 98% purity, would suggest a technical material has been used, so there is uncertainty in actually what has been tested in the study. The tests were conducted according to EPA Guideline "Methods of Measuring the Acute Toxicity of Effluents and Receiving Water to Freshwater and Marine Organisms". However, the origin of the organisms is not given. Therefore, previous exposure the test species may have had to pesticides or other chemicals is unclear. Furthermore, there was no analytical verification of test concentrations reported and the study is non-GLP, thus the reliability of the endpoint is questionable. Given the uncertainty in what was actually tested, the calculated endpoints and the conduct of the test, the study is considered unreliable.

Further points of clarification:
The original Roundup formulation contains POEA surfactant which is not permitted in formulated herbicidal products in the EU. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with high level of caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
This study aims to assess the acute toxicity of Roundup® on two species of the most common freshwater crustaceans in Sri Lanka: calanoid copepod (*Phyllodiaptomus annae* Apstein) and decapod shrimp (*Caridina nilotica* P. Roux).

LC50 for adult copepod *Phyllodiaptomus annae* was determined to be 1.059 mg/L however only 19% mortality was observed at the highest exposure concentration in the test (1.6 mg/L). For the second species, the 72 and 96 hour LC50 for decapod shrimp *Caridina nilotica* was determined to be 107.53 and 60.97 mg/L, respectively. However, the mean percentage mortality at both timepoints was identical from Table 1 in the paper. As there are no biological data presented in the paper, the observed mortality and the LC50 calculation cannot be confirmed. Besides, the percentages mortality values presented do not corroborate the calculated LC50 values (max 53% mortality at 160 mg/L).
The results are unreliable.

The test substance was identified only as 'Roundup'. Given the date of the study, it is likely that the surfactant polyethoxylated tallow amine (POEA) was used in the formulation. The applicant confirms this. Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Effects were observed even at low concentrations however RMS considers that this study is of low reliability and cannot be taken into account as critical information for the assessment of the active substance glyphosate itself.
Due to potential presence of POEA and the doubtful results, the study is considered not relevant and not reliable by RMS.

Data point:	CA 8.2
Report author	Li Jia et al.
Report year	2017
Report title	Acute toxicity study of glyphosate and cyhalofop-butyl to Daphnia carinata.
Document Source	Acta Prataculturae Sinica (2017), Vol. 26, No. 9, pp. 148
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The acute effects of glyphosate on the movement, survival, and phototaxis of *Daphnia carinata* was investigated. There were 5 test concentrations at 12.30, 19.68, 31.57, 50.43 and 80.77 mg/L as well as a control. Every treatment contained 6 replicates with 10 healthy *D. carinatas* each (60 daphnids each treatment). The assessment of survival and activity of the daphnids was observed after 12, 24, 36, 48,

72 and 96 hours, respectively. Furthermore, effect on the phototaxis of *Daphnia carinatas* was evaluated after an exposure of three hours.
Glyphosate had a dose-effect relation to the moving inhibition and fatality rate of *Daphnia carinatas* in the acute toxicity test. Regarding the effects on the survival and activity the LC_{50} values after 24, 36, 48, 72 and 96 hours were 66.58, 58.13, 29.60, 18.83 and 12.33 mg/L, respectively. Regarding the effects on the activity ability the EC_{50} values after 24, 36, 48, 72 and 96 hours were 45.24, 42.49, 26.53, 17.14, and 11.58 mg/L respectively. The concentration of glyphosate causing significant changes in the phototaxis index after 3 hours was 4.59 mg glyphosate/L.

Materials and Methods

Test item
41 % glyphosate isopropylamine saline water agent was purchased from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences and stored at 4-10 °C in the refrigerator.

Daphnia
The original species were collected from the natural water body of the three Gorges Reservoir area of Chongqing and monoclonal biological strains were obtained after multiple generations of parthenogenesis and purification. *Daphnia carinata* was cultured with tap water after full aeration and dechlorination, fed every morning through the 4th day for use with fresh *Scenedesmus obliquus*, a light intensity of 12001x at a photoperiod of 12 h/ d and a temperature at 20 ± 0.5 °C. *Daphnia carinata* with selective breeding and strong phototaxis were selected as the experimental material.

Experimental water
The diluted water (IS06341-1989) artificial standard water (hereinafter referred to as standard water). Its composition is 0.294 g/L $CaCl_2 \times H_2O$, 0.123 g/L $MgS0_4 \times 7 H_2O$, 0.065 g/L $NaHCO_3$, 0.006 g/L KCl. Then the deionized water (20 °C) was pre-oxygenated and the pH was adjusted to 7.80 with 1 mol/L HCl or NaOH. After a session of 24 hours, the deionized water was used.

The effect of herbicides on the survival rate and moving abilities of Daphnia carinata
The concentration of glyphosate was set at 0 (control), 12.30, 19.68, 31.57, 50.43, 80.77 mg/L in a single-factor randomized design. Every treatment contained 6 replicates with 10 healthy *Daphnia carinatas* each (60 daphnids each treatment). The experiment was conducted under the condition of intensity of light 12001 x, photoperiod 12 hours light and the temperature at 20 ± 0.5 °C).

Before starting exposure in 10.0 mL glyphosate of different concentrations, four-day-old daphnids were wetted and washed in the 20.0 mL ISO standard water three times, each time lasting for 5 minutes. The assessments on survival and activity of the *Daphnia carinatas* were made after 12, 24, 36, 48, 72 and 96 hours. Daphnids have been defined as inhibited when by slightly rotating the solution, the tentacles are movable but the individual daphnid cannot swim. Daphnids have been defined as dead when the daphnid sinks into the bottom of the water and has no reaction when the container is slightly rotated.

The effect of herbices on the phototaxis of Daphnia carinatas
10 *Daphnia carinatas* were randomly selected for each repetition. They were put in 100 mL glyphosate solutions of different concentrations for three hours with 12001x intensity of light. Then the *Daphnia carinatas* were transferred to the phototaxis measuring device for measuring the phototaxis. After 5 minutes darkness adaptation, the organisms were stimulated for 10 minutes under the intensity of light of 12001x. From the sixth minute on, the number of *Daphnia carinatas* in each light area was recorded for each minute.
The calculation formula for the phototaxis index of *Daphnia carinatas* (phototaxical index, I_p) was as follows:

$$I_p = (U-L)/(U+M_1+M_2+L)$$

where U, M_1, M_2, and L represent the number of the *Daphnia carinatas* in the upper, middle and upper, middle and lower, and lower part of the observation device, respectively.

Statistics
Excel 2013 was used to do basic calculations and tabulations for the experimental data: SigmaPlot 12.5 software was used for toxicity equation fitting; SPS 17.0 software was used to make variance analysis. The Method of Duncan was adopted to make multiple comparisons, and the significance level was set to $p < 0.05$.

Results

The lethal effect on Daphnia carinatas
Glyphosate showed an apparent dose-effect relationship. The LC_{50} values of glyphosate to *Daphnia carinata* after 24, 36, 48, 72 and 96 hours were 66.58, 58.13, 29.60, 18.83 and 12.33 mg/L, respectively. LC_{50} (24 h) was 2.3 times and 5.4 times of LC_{50} (48 h) and LC_{50} (96 h), respectively, glyphosate LC_{50} decreased as time went on (Table 1).

Table 1: Effect of glyphosate on the death rate of *Daphnia carinata*.

除草剂 Herbicide	时间 Time (h)	LC_{50} (mg/L)	95%置信区间 95% confidence interval (mg/L)	毒性方程 Toxicity equation	R^2	P
草甘膦 Glyphosate	24	66.58a	58.63～75.60	$y=0.63/\{1+\exp[-(x-43.46)/6.72]\}$	0.879	0.000
	36	58.13a	52.64～64.19	$y=1.01/\{1+\exp[-(x-45.58)/7.08]\}$	0.872	0.000
	48	29.60b	26.26～33.38	$y=1.01/\{1+\exp[-(x-30.96)/4.82]\}$	0.876	0.000
	72	18.83c	17.07～20.77	$y=1/\{1+\exp[-(x-19.68)/3.21]\}$	0.891	0.000
	96	12.33c	11.28～13.48	$y=1/\{1+\exp[-(x-12)/2.25]\}$	0.890	0.000

LC_{50}: Different letters in the same column indicate significant difference ($P < 0.05$)
y: cumulative lethality (%)
x: concentration of glyphosate.

Inhibitory effect of herbicides on the activity ability of Daphnia carinatas
Glyphosate significantly reduced the activity ability of *Daphnia carinatas* with an apparent dose-effect relationship. The EC_{50} values of glyphosate to *Daphnia carinata* at 24, 36, 48, 72 and 96 h were 45.24, 42.49, 26.53, 17.14, and 11.58 mg/L respectively. The EC_{50} of glyphosate decreased significantly as time went on (Table 2).

Table 2: Effect of glyphosate on the movement of *Daphnia carinata*.

除草剂 Herbicide	时间 Time (h)	EC_{50} (mg/L)	95%置信区间 95% confidence interval (mg/L)	毒性方程 Toxicity equation	R^2	P
草甘膦 Glyphosate	24	45.24a	40.81～50.15	$y=0.9/\{1+\exp[-(x-42.64)/5.25]\}$	0.893	0.000
	36	42.49a	38.47～46.93	$y=1/\{1+\exp[-(x-44.23)/7.28]\}$	0.878	0.000
	48	26.53b	23.36～30.13	$y=1/\{1+\exp[-(x-27.86)/5.21]\}$	0.877	0.000
	72	17.14c	15.47～18.99	$y=1/\{1+\exp[-(x-17.84)/3.38]\}$	0.890	0.000
	96	11.58c	10.64～12.61	$y=1/\{1+\exp[-(x-10.66)/2.36]\}$	0.891	0.000

EC_{50}: Different letters in the same column indicate significant difference ($P < 0.05$)
y: cumulative inhibition (%)
x: concentration of glyphosate

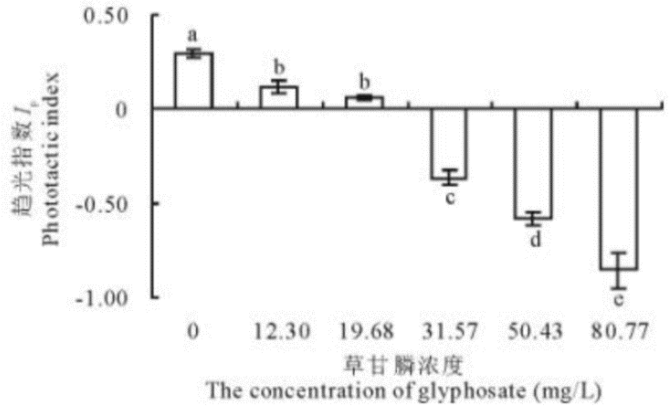
The effect of herbicides on the phototaxis of Daphnia carinatas
With increasing glyphosate concentrations, the phototaxical index (Ip) of *Daphnia carinatas* decreased significantly. The phototaxical index (Ip) in the control was 0.3. In the glyphosate treatments, the

relationship of *Daphnia carinatas* phototaxical index (Ip) and the concentration of glyphosate was expressed by the equation:

$y = 10\text{-}_4x_2 - 0.023x + 0.355$, $R_2 = 0.937$
(y represents I; x represents the concentration of glyphosate mg/L)

At the glyphosate concentration of 4.59 mg/L (1/1787 of the application concentration) the phototaxical index (Ip) significantly decreased. When the concentration of glyphosate increased from 19.68 mg/L to 31.57 mg/L, the phototaxical index (Ip) turned from positive to negative. At the glyphosate concentration of 80.77 mg/L, the phototaxis of *Daphnia carinatas* was extremely weak, and the phototaxical index (Ip) was determined to be -0.858. Therefore the phototaxis had decreased by 1.158 units of the the phototaxical index compared to the control group (Fig. 1).

Figure 1: Effects of glyphosate on the phototaxis of *D. carinatas*



The EC_{50} (24 h) and LC_{50} (24 h) of the daphnia were 45.24 and 66.58 mg glyphosate/L, respectively. But at 3 hours, the concentration that made Ip have a significant change was only 4.59 mg glyphosate)/L.

Conclusion

Glyphosate had a dosage-effect relation to the moving inhibition and fatality rate of *Daphnia carinatas* in the acute toxicity test. Regarding the survival and activity the LC_{50} after 24, 36, 48, 72 and 96 hours were 66.58, 58.13, 29.60, 18.83 and 12.33 mg/L, respectively. Regarding the activity ability the EC_{50} after 24, 36, 48, 72 and 96 hours were 45.24, 42.49, 26.53, 17.14, and 11.58 mg/L respectively. The concentration of glyphosate causing significant changes in the phototaxis index after 3 hours was 4.59 mg/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The herbicides evaluated in the study were a 41% glyphosate isopropylamine saline water agent (this formulation is not representative for the glyphosate EU renewal, the representative formulation is MON 52276). The study was not conducted according to GLP and the test substance source could not be verified. The authors state that glyphosate has an obvious dose-effect relation to the moving inhibition and fatality rate of *Daphnia carinatas*. The routinely used concentration of the two is significantly higher than the LC_{50} and is strongly toxic to *Daphnia carinatas*. However, given the lack of standard guidelines, an unclear method design and approach, as well as challenges in interpreting the study results make reaching any conclusions arising from the study challenging at best.

Further points of clarification:
The age of the animals at the start of the test is not stated. The environmental conditions during the exposure phase were not recorded. Chemical analysis was not performed and therefore exposure cannot be confirmed.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
Original report in Chinese, results are based on a document translated in english.
The acute effects of glyphosate (in a formulation not identified) on the movement, survival, and phototaxis of Daphnia carinata were investigated.
LC50 values after 24, 36, 48, 72 and 96 hours were 66.58, 58.13, 29.60, 18.83 and 12.33 mg/L, respectively. Regarding the effects on the activity ability the EC50 values after 24, 36, 48, 72 and 96 hours were 45.24, 42.49, 26.53, 17.14, and 11.58 mg/L respectively.

No information on surfactants were provided. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. No analytical verification is available. Only LC50/EC50 are presented (no details on the results are provided). RMS considers that this study is not reliable and cannot be taken into account as critical information or the assessment of the active substance glyphosate itself.

Less relevant but supplementary (uncertainty on the test item) and not reliable.

Data point:	CA 8.2.4
Report author	Cordova Lopez A. M. *et al*.
Report year	2019
Report title	Exposure to Roundup® affects behaviour, head regeneration and reproduction of the freshwater planarian *Girardia tigrina*
Document Source	Science of the total environment (2019), Vol. 675, pp. 453
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: This is an invasive flatworm species in the EU. No specific test guidelines are available for this type of study, despite the range of endpoints that appear to have been covered.

Further points of clarification:
Roundup Original is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). Moreover, it contains POEA surfactants that is not permitted for use in formulated herbicidal products in the EU. The purity of the formulation and the identity of co-formulants was not confirmed by the authors. Therefore, the findings of this paper should be treated with a high level of caution as the influence of POEA surfactant on the achieved findings cannot be excluded.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
Acute and chronic toxicity of Roundup® on *Girardia tigrina* (freshwater planarian) were assessed. Locomotor velocity, feeding rate, regeneration, reproductive parameters and morphological abnormalities of *Girardia tigrina* were impaired. This species is considered invasive in Europe.
For acute exposure, a 48 h LC50 of 35.94 mg glyphosate/L was derived. The LOEC feeding, locomotion, regeneration was 3.75 mg glyphosate/L.
For exposure to glyphosate over 5 weeks, EC50 was 1.6 mg glyphosate/L for fecundity and fertility rates. The LOEC was 1.87 mg glyphosate/L.

Roundup Original was used. The applicant indicated that it is not the EU representative formulation MON52276. According to the applicant it contains POEA surfactants. Besides, in an other study (from Brazil), Sanchez et al 2017 states that « It is known that the surfactant MON 0818, containing POEA, integrates the Roundup Original formulation. The MON 0818 (a code of Monsanto for designation for preparation of POEA) is a mixture of polyethoxylated long-chain alkylamines synthesized from animal-derived fatty acids and is added to facilitate glyphosate penetration into the plants."

This indicates the presence of POEA in the formulation tested.

POEA is not authorized in plant protection products containing glyphosate (European Commission, August 2016).
Due to presence of POEA, the study is considered not relevant by RMS. The reliability of the study was not assessed.

Data point:	CA 8.2.4.2, CA 8.2.5.2
Report author	Liu Xiao-wei et al.
Report year	2012
Report title	Toxicological effect of paraquat and glyphosate on cladoceran *Moina macrocopa*.
Document Source	Shengtaixue Zazhi (2012), Vol. 31, No. 8, pp. 1984-1989
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The acute toxicities of glyphosate to the Cladoceran *Moina macrocopa* as well as the sub-lethal concentrations of glyphosate (0, 0.4, and 1.6 mg/L) on the life-table demography were studied.
The 48 hour-LC_{50} value of glyphosate to *Moina macrocopa* was 26.287 mg/L under the condition of feeding of 1.0×10^6 cells/mL *Scenedesmus obliquus*. Glyphosate concentrations had a significant effect on life expectation, generation time and population intrinsic growth rate of *Moina macrocopa* ($P < 0.01$). Glyphosate at concentrations of 0.4 and 1.6 mg/L significantly shortened the life expectancy of *Moina macrocopa*. The net reproductive rate and population intrinsic growth rate of *Moina macrocopa* was not significantly affected at 0.4 and 1.6 mg glyphosate/L.

Materials and methods

The source and culture of Moina macrocopa
The *Moina macrocopa* used in the lab were obtained from Nagasaki University of Japan. In the laboratory, it was cloned and cultured at 25 ±1 °C and under natural light for more than 3 months. The culture medium was aerated tap water. The bait was cultured from HB-4 medium in an exponential growth period of *Scenedesmus obliquus*, centrifuged and concentrated, and used after counting. Prior to the experiment, *Moina macrocopa* was pre-cultured. During the pre-culture, 1.0×10^6 cells/mL *Scenedesmus obliquus* was fed daily and half of the *Moina macrocopa* cultures were replaced.

Preparation of the test solution
Glyphosate (Huizhou Zhongxun Chemical Co., Ltd.) was purchased in Wuhu pesticide market, and the solutions were prepared through mother liquor dilution method. The mother liquor of 10 mg/L was prepared in distilled water and stored in a refrigerator at 4 °C. The mother liquor was prepared once in 3 days, and the required concentration of the test solution was prepared with aerated tap water.

Acute toxicity testing
The *Moina macrocopa* used in the experiment was a larva after three generations of parthenogenesis under standard experimental conditions. The experiment was carried out in a 50 mL beaker with

10 *Moina macrocopa* (age < 10 hours) and 50 mL test solution (containing *Scenedesmus obliquus* with the density of 1.0×10^6 cells/mL) added in each beaker.
The concentration of glyphosate was: 5.6, 10.0, 18.0, 32.0 and 56.0 mg/L plus a blank control group. 3 replicates were set up in each group.
Assessments of number of mortalities of *Moina macrocopa* were made 48 hours later in each beaker. Individuals were considered as dead when turning the test container by hand and *Moina macrocopa*, sank to the bottom of the container within 15 seconds.
Afterwards the 48 h LC_{50} value was calculated by probability unit method.

Chronic toxicity test
The tested glyphosate concentration were 0.4 and 1.6 mg/L. Each group consisted of 3 replicates, each with 10 individuals aged <10 hours. The test solution contained 1.0×10^6 cells/mL. After test start, the survival and production of *Moina macrocopa* were observed every day. The number of larvae was transferred to a vessel containing fresh test fluid (*Scenedesmus obliquus* with a density of 1.0×10^6 cells/mL), and the test lasted until all the subjects died.
Individuals were considered as dead if the heart beat of *Moina macrocopa* was stopped (observed under anatomic microscope). At test end, based on the age-specific survival and reproduction rates, the vital statistical parameters such as life expectancy, reproductive rate, generation time and intrinsic growth rate were calculated.

Data Processing
SPSS 17.0 was used to analyse the test data. After the normality test, the significant differences between each concentration group and the blank control group were analysed by two-factor variance analysis and multiple comparison (LSD test).

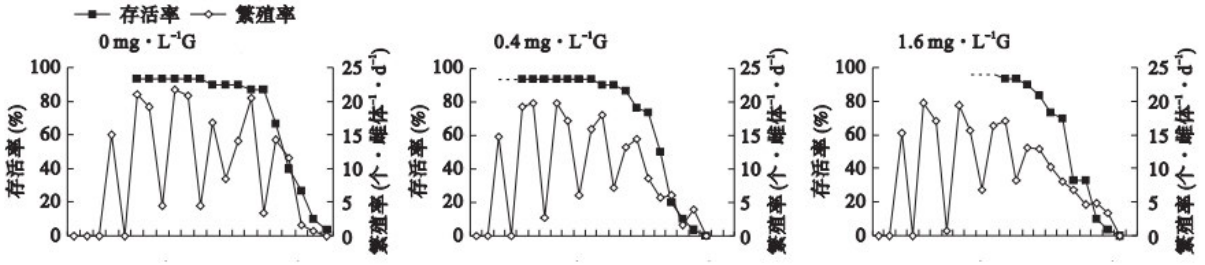
Results

Single acute toxicity of glyphosate to Moina macrocopa
There was a significant dose-effect relationship between the mortality of *Moina macrocopa* and the concentration of glyphosate at 48 hours. The 48-h LC_{50} (95 % CI) value of glyphosate to *Moina macrocopa* was 26.287 mg/L (22.945 - 30. 216 mg/L).

Effect of glyphosate on the survival rate and reproduction rate of Moina macrocopa
The increase of glyphosate concentration had no significant effect on the survival rate and the breeding rate of *Moina macrocopa*.

The age-specific survivorship and fecundity of *Moina macrocopa* exposed to glyphosate concentrations at 0.4 and 1.6 mg/L is shown in the figures below.

Figure 1. Age-specific survivorship and fecundity of *Moina macrocopa* exposed to the control and the glyphosate concentrations of 0.4 and 1.6 mg/L (G: glyphosate)



Effect of glyphosate on statistical parameters of life table of Moina macrocopa
*T*he statistical parameters of the life table of *Moina macrocopa* in different concentrations of glyphosate are shown in Table 1.

Table 1. Life-table demographic parameters of *Moina macrocopa* exposed to different glyphosate concentrations

Parameters	Glyphosate concentration ($mg \cdot L^{-1}$)		
	0	0.4	1.6
Life expectancy (d)	15.97±0.29	15.97±1.86	17.23±1.00
Generation time (d)	226.19±4.23	228.43±4.79	235.32±5.83
Net breeding rate (ind.)	170.72±3.38	165.53±19.16	174.18±12.25
Innate/intrinsic rate of increase (d^{-1})	0.97±0.02	0.97±0.01	0.99±0.01

* indicating a significant difference from the control group ($P < 0.05$), and the data were mean ± standard errors

Glyphosate concentration had a significant effect on life expectation, generation time and population intrinsic growth rate of *Moina macrocopa* ($P < 0.01$) (Table 2). Glyphosate at concentrations of 0.4 and 1.6 mg/L significantly shortened the life expectancy of *Moina macrocopa*.

Table 2. Two-way ANOVA for the effects of glyphosate on the life table demographic parameters of *Moina macrocopa*

Parameters	*df*	*MS*	*F*	*P*
Life expectancy (d)	2	7.194	7.302	<0.01
Generation time (d)	2	4377.755	41.833	<0.01
Net breeding rate (ind.)	2	553.667	3.478	>0.05
Innate/intrinsic rate of increase (d^{-1})	2	0.014	6.642	<0.01

* indicating a significant difference from the control group ($P < 0.05$), and the data were mean ± standard errors

Conclusion

In this acute toxicity test, the 48 hour LC_{50} value of glyphosate to *Moina macrocopa* was 26.287 mg/L under the condition of feeding 1.0×10^6 cells/mL *Scenedesmus obliquus*. Glyphosate concentrations had a significant effect on life expectation, generation time and population intrinsic growth rate of *Moina macrocopa* ($P < 0.01$). Glyphosate at concentrations of 0.4 and 1.6 mg/L significantly shortened the life expectancy of *Moina macrocopa*. The net reproductive rate and population intrinsic growth rate of *Moina macrocopa* was not significantly affected at 0.4 and 1.6 mg glyphosate/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The conclusions of the study are unclear based on several factors including the impact of the density of the algal food source and the temperature of the test media. This study is not adequately described – for example, water quality / environmental conditions cannot be confirmed. There were no validity criteria stated and no analytical verification of exposure concentrations was undertaken. Given the uncertainty over the test design and the procedures undertaken and the fact that the study was not conducted according to a recognised test guideline relevant for the EU ecotoxicological regulatory risk assessment, the study is considered unreliable.

Assessment and conclusion by RMS:

Original report in Chinese, results are based on a document translated in english.
It was not based on a relevant guideline (no guideline available). Only LC50 is available, no biological data were presented. No NOEC was derived.
This study is not adequately described. There were no validity criteria stated and no analytical verification of exposure concentrations was undertaken. Inconsistencies are noted in the translated report e.g. LC50 reported in the summary does not correspond to the one given in the results chapter.
The study is relevant but not reliable.

Data point:	CA 8.2.4
Report author	Ruiz-Gonzalez E. L. et al.
Report year	2018
Report title	Assessment of median lethal concentration (CL50) of pollutants on Macrobrachium tenellum juveniles
Document Source	Latin American Journal of Aquatic Research (2018), Vol. 46, No. 3, pp. 589 - 592
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The toxicity of glyphosate-based herbicide on fresh-water prawn *Macrobrachium tenellum* juveniles were investigated. There were 8 organism per experimental unit. All bioassays were performed with 4 replicates plus a negative control. Dead individuals were counted at the end of 24 hours of exposure.

In the acute toxicity test with fresh-water prawn *Macrobrachium tenellum* juveniles the 24 hours-LC_{50} of glyphosate-based herbicide was 32 ± 6 mg/L.

Materials and methods

Test item
Faena Clásico® : contains less than 35.6 % glyphosate potassium salt.

Test organisms
Juveniles of *Macrobrachium tenellum* were used in a size range of 2-4 cm in length, measured from the tip of the rostrum at the rear end of the telson. Individuals were collected from the laboratory stock.

Test design
The concentrations tested were 180, 90, 45, 22.5 and 11.2 mg/L.
8 individuals were placed per experimental unit, which consisted of PET containers with a capacity of 2 L, which were covered with a mesh to prevent escape.
The record of dead individuals was registered every 6 h for 24 h of exposure. The organisms were considered dead when no response to stimuli, or movement of their appendix and in most cases, individuals showed an opaque white colour.
All bioassays were performed with 4 replicates plus a control without treatment. The bioassays were carried out at room temperature of 28 °C and water conditions prior the addition of compounds was pH 7.5 and dissolved oxygen 5 mg/L.

Statistics
The mean lethal concentration (LC_{50}) was calculated in the IBM® SSPS® Statics Software, as described by Finney. In order to determine the standard error, Randhawa (2009) instructions were followed.

Results

The average lethal concentration (LC_{50}) of glyphosate-based herbicide on *Macrobrachium tenellum* juveniles after 24 hours of exposure was 32 ± 6 mg/L.

Conclusion

In the acute toxicity test with fresh-water prawn *Macrobrachium tenellum* juveniles the 24 hour-LC_{50} of glyphosate-based herbicide was 32 ± 6 mg/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Considered supplementary as the test substance cannot be explicitly identified. Information presented suggests that this is not the representative formulation for the glyphosate EU renewal as it is based on the potassium salt of glyphosate.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
The toxicity of glyphosate-based herbicide on fresh-water prawn Macrobrachium tenellum juveniles were investigated. In the acute toxicity test with fresh-water prawn Macrobrachium tenellum juveniles the 24 hours-LC50 of glyphosate-based herbicide was 32 ± 6 mg/L.
No specific guideline was followed.
No biological data are reported.
No sufficient details on the test conditions are reported.
The study is less relevant but supplementary and considered not reliable for regulatory risk assessment.

Data point:	CA 9
Report author	Magbanua F. S. *et al.*
Report year	2013
Report title	Individual and combined effects of fine sediment and the herbicide glyphosate on benthic macroinvertebrates and stream ecosystem function
Document No	Freshwater biology (2013), Vol. 58, No. 8, pp. 1729-1744
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Paper describes a specific multiple mesocosm study conducted in New Zealand - a study which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices, non-EU monitoring data, residue definitions differing from EU). In addition, the study was conducted with a glyphosate formulation that contained polyethoxylated tallowamine (POEA) surfactant that is not permitted for use in agrochemical formulations in the EU. As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal. The surfactant system used in the formulation tested in this paper is POEA based, whereas the surfactant used in MON 52276 is quaternary-ammonium based. The article is therefore not relevant to the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

In this study, it is clearly stated by the study authors that the formulation included the surfactant polyethoxylated tallow amine (POEA). Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Therefore it is not possible to discriminate between glyphosate and POEA.
The study is considered not relevant by RMS.

Data point:	CA 9
Report author	Reno U. *et al.*
Report year	2018
Report title	Effects of glyphosate formulations on the population dynamics of two freshwater cladoceran species.
Document No	Ecotoxicology (2018), Vol. 27, No. 7, pp. 784-793
Guidelines followed in study	OECD 204, ASTM 1980, APHA 1998
Deviations from current test guideline	Not mentioned
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The general objective of this work was to experimentally assess the effects of acute glyphosate pollution on two freshwater cladoceran species (*Daphnia magna* and *Ceriodaphnia dubia*) and to use this information to predict the population dynamics and the potential for recovery of exposed organisms. Five to six concentrations of four formulations of glyphosate (4-Gly) (Eskoba®, Panzer Gold®, Roundup Ultramax® and Sulfosato Touchdown®) were evaluated in both cladoceran species through acute tests and 15-day recovery tests in order to estimate the population dynamics of microcrustaceans. The endpoints of the recovery test were: survival, growth (number of molts), fecundity, and the intrinsic population growth rate (r). A matrix population model (MPM) was applied to *r* of the survivor individuals of the acute tests, followed by a Monte Carlo simulation study. Among the 4-Gly tested, Sulfosato Touchdown® was the one that showed higher toxicity, and *C. dubia* was the most sensitive species. The Monte Carlo simulation study showed an average value of λ always <1 for *D. magna*, indicating that its populations would not be able to survive under natural environmental conditions after an acute Gly exposure between 0.25 and 35 a.e. mg L^{-1}. The average value of λ for *C. dubia* was also <1 after exposure to Roundup Ultramax®: 1.30 and 1.20 for 1.21 and 2.5 mg a.e. L^{-1}, respectively. The combined methodology-recovery tests and the later analysis through MPM with a Monte Carlo simulation study-is proposed to integrate key demographic parameters and predict the possible fate of microcrustacean populations after being exposed to acute 4-Gly contamination events.

Materials and methods

The 4-Gly formulations selected were Eskoba® (Red Surcos), Panzer Gold® (Dow AgroSciences), Roundup Ultramax® (Monsanto) and Sulfosato Touchdown® (Syngenta Agro). The 4-Gly contained: 48% (w/v) isopropylamine salt; 60.8% (w/v) dimethylamine salt; 74.7% monoammonium salt; and 62% (w/v) potassium salt, respectively.

Acute test

Before the tests, the researcherspreparedsolutions of 1000 mg a.e./L of the 4-Gly in sterile bidistilled water maintained under constant conditions of darkness at -4 °C until their analytic determination through high-performance liquid chromatography (HPLC) using a Dionex DX-100 ion chromatograph. The concentrations of glyphosate in the solutions were: 1067; 1091; 914 and 993 mg a.e./L in the formulations of Eskoba®, Sulfosato Touchdown®, Panzer Gold® and Roundup Ultramax® respectively. This stock solution was diluted in synthetic culture medium to prepare each one of the test concentrations to be used in the experiments with the two cladoceran species.

The stock cultures of *D. magna* were maintained in synthetic media containing 0.13 g K_2SO_4, 1.12 g $CaCl_2$, 1 g $NaHCO_3$ dissolved in 5 L of distilled water and *C. dubia* in the synthetic media: 2.4 g $MgSO_4$, 3.84 g $NaHCO_3$, 0.16 g KCl, and 2.4 g $CaSO_4$ * $2H_2O$ dissolved in 20 L of distilled water. These media were used to do all the tests (acute and recovery ones). The cladocerans were fed with 40 µL (absorbance = 1.5 λ, 650 nm) of *Chlorella vulgaris* (strain CLV2, taken by the CISECE, Mexico) per organism and maintained in cultivation chambers under controlled and constant conditions: photoperiod 16 L: 8D and temperature 20 ± 1 °C. The 48-h acute tests were performed with photoperiod 16 L: 8D and temperature 20 ± 1 °C. Twenty neonates (<24 h) of *C. dubia* and *D. magna* were divided into four groups of five specimens for each one of the treatments and controls.

For the commercial formulation Panzer Gold the researchers tested 5 concentrations of glyphosate: 10, 5, 2.5, 1.25, 0.62 mg a.e./L for *D. magna* and 6 concentrations for *C. dubia*: 2.5, 1.25, 0.62, 0.49, 0.31, and 0.15 0.62 mg a.e./L. For Roundup Ultramax the researchers tested 5 concentrations of glyphosate: 40, 20, 10, 5, 2.5 mg a.e./L for *D. magna*, and 20, 10, 5, 2.5, 1.25 mg a.e./L for *C. dubia*. The concentrations tested and the 48 h-LC_{50} for Eskoba and Sulfosato Touchdown have already been reported by Reno et al. (2015). The mortality was recorded as an indicator of acute toxicity and assessed by the immobilization of the organisms. As indicative of the toxic effect, in this work the researchers considered the complete immobilization of the organisms and the absence of response after being gently stimulated or pushed by a metal rod. The results were considered acceptable when the mortality in the control was ≤10%. For each species, the researchers determined the lethal concentration 50 at 48 h (48

h-LC_{50})- The values of 48 h-LC_{50} with a 95% confidence level were determined by the probit analysis. The pH and the dissolved oxygen (DO, mg/L) were measured at the beginning and at the end of all tests. The values were between 7.6 and 8.1 for the pH, and 6 and 8 mg/L for DO, within the normal limits.

Recovery test
To estimate the post-exposure population dynamics in the 4-Gly formulations and concentrations tested, recovery tests (RTs) were performed, following the procedure proposed by Reno et al. (2014): Each surviving individual of the acute tests was placed alone in a new 50 ml glass vessel with the respective glyphosate-free culture media for 15 days under the same conditions described for stock cultures (photoperiod 16 L: 8D and temperature 20 ± 1 °C). The cladocerans were fed three times a week, with 40µ (absorbance = 1.5 λ, 650 nm) of a suspension of *C. vulgaris*.
At the same time, data were taken from each vessel, to determine the endpoints: survival (days), number of molts produced and released in the vessels (as indirect measure of growth, since the number of molts increases with body size), and fecundity (number of neonates released).
The values of the pH and the concentrations of DO were recorded at the beginning and at the end of each test, controlling that they were within the limits.

Construction of the matrix population models
With the results obtained from the RTs, the researchers constructed matrix population models (MPM). These models have been used with good results to evaluate chronic effects of agricultural chemicals on non-target species (Forbes et al. 2016). In the matrix population models, the self-values of higher module (called λ) are related to the asymptotic dynamics of the population. The logarithm of λ is the intrinsic population growth rate (*r*). That means that the population grows if the module of λ is higher than 1 (or r > 0) (Santadino et al. 2014).

Monte Carlo simulation
The researchers performed a Monte Carlo simulation study of the matrix population models to estimate the probability distribution of the asymptotic population growth rate for each treatment and control. For each random matrix, the researchers computed the dominant eigenvalue, thus obtaining a 106 sample from the probability distribution of the projected population growth rate corresponding to each treatment. The mean and 95% equal-tailed probability interval for the growth rate of each treatment were computed using the empirical distribution functions. The probability density function of the population growth rate was also estimated by a kernel diffusion method (Botev et al. 2010). All Monte Carlo computations were performed using the free software GNU Octave 3.8.2 (Octave community 2014). The probability density estimation was computed using the code provided by Botev et al. (2010).

Results

Acute test
Table 1 shows the values of 48 h-LC_{50} for the four glyphosate-based formulations studied. The values of 48 h-LC_{50} corresponding to Sulfosato Touchdown were lower for both cladoceran species, *C. dubia* being the most sensitive one. The specimens surviving the acute tests were used in the recovery tests.

Table 1. Effective concentrations for 50% of the population (LC_{50} mg e.a./L) obtained after 48 h exposure of *D. magna* and. *C. dubia* to the four glyphosate-based formulations tested. In brackets, limits of confidence (α = 0.05)

Glyphosate-based formulations	48 h-LC_{50} (mg a.e. L^{-1})		References
	D. magna	*C. dubia*	
Eskoba®	29.48 (27.464–31.415)	14.49 (12.4–16.77)	Reno et al. (2015)
Roundup Ultramax®	11.68 (8.93–15.43)	4.84 (3.83–6.13)	In this work
Panzer Gold®	2.12 (1.68–2.67)	0.54 (0.44–0.65)	In this work
Sulfosato Touchdown®	1.62 (1.24–2.09)	0.31 (0.25–0.37)	Reno et al. (2015)

Recovery tests

The organisms that survived until the end of the recovery test were previously exposed to the following concentration of glyphosate: Eskoba: 35, 30, 25, 20 mg a.e./L for *D. magna*, and 18, 12, 8 mg a.e./L for *C. dubia*. Sulfosato Touchdown: 0.5, 0.25 mg a.e./L for *D. magna*, and 0.25, 0.125, 0.0625 mg a.e./L for *C. dubia*. Panzer Gold: 2.5, 1.25, 0.625 mg a.e./L for *D. magna*, and for *C. dubia*: 0.625, 0.49, 0.3125, 0.156 mg a.e./L. Roundup Ultramax: 10, 5, 2.5 mg a.e./L for *D. magna*, and 2.5, 1.25 mg a.e./L for *C. dubia*. In relation to the results of the Monte Carlo simulation, the average value of λ for *D. magna* exposed to all the formulations and concentrations evaluated was always lower than 1, so under these conditions, the population will not be able to survive over time after an acute exposure to the concentrations tested in this work. In the case of *C. dubia*, the average value was also lower than one, except in the assays carried out with Roundup Ultramax, where λ was 1.30 and 1.20. At both concentrations tested, the results were similar to 0.15 mg a.e./L (λ = 1.05) of Panzer Gold and to 0.06 mg a.e./L (λ = 1.01) of Sulfosato Touchdown. Furthermore, different neonatal mortality rates were recorded in the recovery tests for the two more toxic formulations: Panzer Gold and Sulfosato Touchdown (Table 2).

Table 2. Mortality of neonates (%) in the recovery tests with *D. magna* and *C. dubia*

	Neonates mortality (%)	
Glyphosate-based formulations (mg a.e. L^{-1})	*D. magna*	*C. dubia*
Panzer Gold®		
2.5	11.1	–
1.25	12.5	–
0.625	14.2	–
0.3125		18.8
0.156	–	28
Sulfosato Touchdown®		
0.25	–	19.7

Conclusion

This study provides relevant information on the toxicity effects of different glyphosate-based formulations on the population dynamics of freshwater microcrustaceans. Since the effect of agricultural pollution at a population level and the potential recovery capacity of the exposed organisms remain poorly studied-partly because of methodological constraints-a methodology of intermediate complexity between the most commonly used acute and chronic tests was proposed. In brief, it consists of performing acute tests followed by recovery tests and a later analysis through MPM with Monte Carlo simulations. It showed to be very useful for integrating key demographic parameters such as survival, growth and fertility. Among the glyphosate-based formulations tested, Sulfosato Touchdown was the most toxic, *C. dubia* being the most sensitive species. This combined methodology-recovery tests

followed by a Monte Carlo simulation study-is proposed to assess ecotoxicity at a population level. It predicts the possible fate of cladoceran populations after being exposed to an acute contamination event. This knowledge can be used in ecotoxicological evaluations and risk assessments and would contribute to environmental management through the establishment of guideline values for the protection of the aquatic biota.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: None of the tested formulations is the representative formulation for the glyphosate EU renewal. Therefore, the article is not relevant for the renewal.

Eskoba® (commercialized by Red Surcos, contains 48% (w/v) isopropylamine salt), Panzer Gold® (commercialized by Dow AgroSciences, contains 60.8% (w/v) dimethylamine salt), Roundup Ultramax® (commercialized by Monsanto, contains 74.7% monoammonium salt) and Sulfosato Touchdown® (commercialized by Syngenta Agro, contains 62% (w/v) potassium salt).

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Acute effects of 4 glyphosate formulations were investigated on two freshwater cladoceran species (*Daphnia magna* and *Ceriodaphnia dubia*). This study aims to predict the population dynamics and the potential for recovery of exposed organisms (using population model).
Eskoba®, Panzer Gold®, Roundup Ultramax® and Sulfosato Touchdown® were used in both cladoceran species through acute tests and 15-day recovery tests in order to estimate the population dynamics of microcrustaceans.
The study reports LC50 values for *Daphnia magna* for the 4 formulations. They all indicate far lower LC50 than those available for the active substance alone. *Ceriodaphnia dubia* seems more sensitive than *Daphnia magna*.
In view of the formulation description, the use of the results to assess the toxicity of glyphosate as formulated in MON52276 is questionable. Only LC50 values are presented, no biological data is available. Analytical verification may not have been conducted. The reliability of the model predictions was not assessed by RMS.
Therefore RMS considers that this study is less relevant but supplementary (due to the different formulation tested) and not reliable.

5. Algae and aquatic plants

Data point:	CA 8.2.7
Report author	de Jesus Veloso Castro A. *et al*.
Report year	2015
Report title	Using a toxicity test with *Ruppia maritima* (Linnaeus) to assess the effects of Roundup
Document Source	Marine pollution bulletin (2015), Vol. 91, No. 2, pp. 506
Guidelines followed in study	None

Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

This study aimed to examine Roundup toxic effects on *Ruppia maritima* specimens collected from Jansen Lagoon (São Luís, MA, Brazil) and acclimatized under laboratory conditions. The numbers of new and dead leaves, the root and leaf length, the chlorophyll *a* content, and the weight of *R. maritima* branches were determined before and after exposure to different Roundup concentrations for seven days. High concentrations caused a significant lethal effect. In addition, significant changes were observed in the wet and dry weights, the number and length of the leaves, and the chlorophyll *a* content. Leaf elongation was observed in the branches exposed to low concentrations, and this change was likely activated as a compensatory mechanism. The results indicate that high concentrations of this herbicide may compromise estuarine flora.

Materials and methods

R. maritima specimens were collected from Jansen Lagoon (São Luís, MA, Brazil) and transported in plastic bags with local water (28 °C, pH 8.87, and 12 g/kg salinity) to the Laboratory of Ecotoxicology (Maranhão Federal University). The specimens were cleaned with freshwater and acclimated for 30 days in a 30-cm-high and 50-cm-diameter container with water (12 g/kg salinity) and beach sand (10-cm layer) from an unpolluted site (Araçagi beach, São José de Ribamar, Maranhão).

The plants were kept in these conditions of cultivation to produce biomass enough for the growth and toxicity tests with Roundup. After acclimatization, morphologically similar, individual apical ramets consisting of a node with the same number of leaves and roots, with approximately 5 cm in length were transferred to 200-ml transparent polyethylene cups (7.0-cm-high) covered with a plastic film. The cups contained 150 ml solution of diluted salt water (12 g/kg salinity) and a blend of commercially available nutritious solutions to aquatic plants (20.0 g/L nitrate and 20.0 g/L phosphate, Aquafauna; 0.68 g/L potassium and 0.48 g/L iron, Flora Pride Tetra). For testing growth and toxicity, no substrates were added in polyethylene cups. The plants were maintained for seven days in a random distribution in a germination chamber that was programmed with a 12-h photoperiod (four 20-W 6500-K fluorescent lamps) and an average inner radiation of 200 µmol/m^2×s and maintained at 25°C. Casting between the cups was performed every day to ensure that all of the plants received the same amount of light. After this period, 12 branches with similar green color, containing roots and four leaves each were transferred individually to new polyethylene cups that contained Roundup solutions (log-dilution) with 0.005, 0.05, 0.5, 5, and 50 mg/L by mixing commercial Roundup with nutritive brackish water in a volumetric flask. The control treatment (only brackish water and nutritive solution) was maintained at the same conditions as described above. After seven days of exposure, pH and salinity were measured, and the number and length of new leaves, the number of dead leaves, the root length, the wet and dry weights, and the chlorophyll a level were measured.

The dry weights of six branches exposed to each solution (including the control) were determined (± 0.001 g) after drying at 60°C to a constant weight. Six other branches were used for the chlorophyll *a* analysis. The water droplets adhered to the branches were removed using absorbent paper, and the wet weight was determined. The branches were then individually homogenized with acetone/water solution (9:1) in a mortar and pestle under ice and protected from light for approximately one minute. The extracted solution was transferred to centrifuge tubes covered with foil and maintained at 4°C for 72 h. The final volume was then adjusted to 4 ml using acetone/water solution (9:1), and the tubes were maintained at room temperature until centrifugation (2000 rpm; 10 min). The absorbance (OD) of the supernatant was determined at 630, 645, 663, and 750 nm. The chlorophyll-a level was calculated using the following formula:

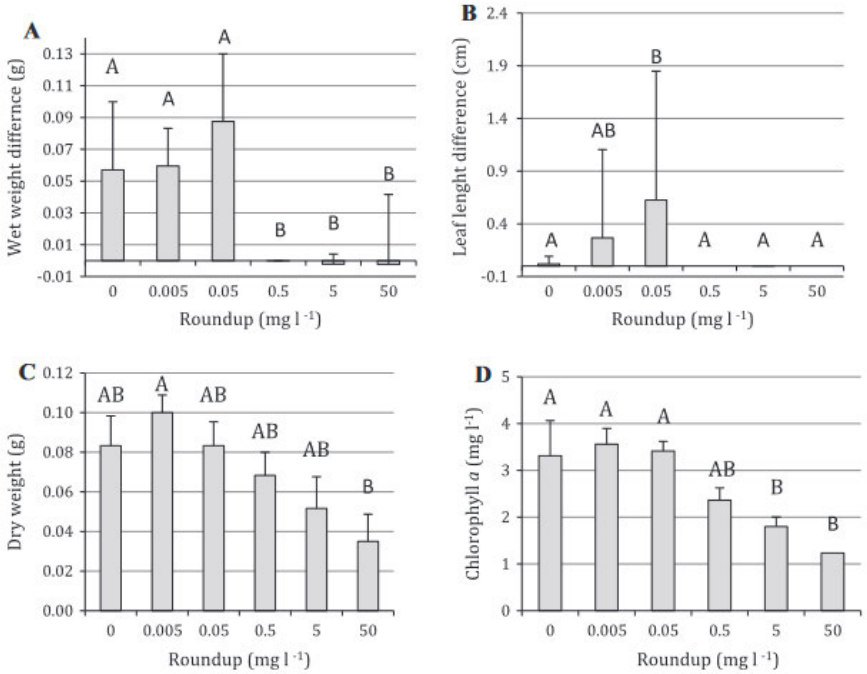
Chlorophyll-a (mg/L) = $11.64 \times OD_{663} - 2.16 \times OD_{645} + 0.10 \times OD_{630} + 9.58 \times OD_{750}$.

Due to the degree of mortality observed in branches subjected to 5 and 50 mg/L treatments at least six branches could be analysed (the minimum number required for statistical analysis). All of the results were submitted to ANOVA followed by Dunnett's test (5%).

Results

The transplant procedures and acclimation produced enough ramifications for all experiments with *R. maritima*. Prior to exposure to Roundup, all of the parameters of the selected branches were similar between the groups (ANOVA, $p > 0.05$). The water parameters (pH and salinity) were similar at the end of exposure (ANOVA, $p > 0.05$).

Figure 1. Differences in the wet weight (A) and leaf length (B) of *Ruppia maritima* before and after exposure to Roundup for seven days. Dry weight (C) and chlorophyll a contents (D) of *Ruppia maritima* after exposure to Roundup for seven days. The error bars represent the standard deviations. The different letters indicate a significant effect (ANOVA-Dunnett's test, $p < 0.05$.



After seven days of exposure to Roundup, a few of the leaves showed remarkable chlorosis, and the number of leaves with chlorosis was significantly higher ($p < 0.05$) in the branches exposed to high concentrations. The branches exposed to 5.0 mg/L (two branches) and 50 mg/L (11 branches) presented low values of the difference of wet weight between the end and the beginning of the experiment (Fig.

1a) and consequent mortality. The only living *R. maritima* at the end of the exposure period to a concentration of 50 mg/L did not produce new leaves.

The analysis of the difference of leaf lengths between the end and the beginning of the experiment revealed foliar elongation in the branches exposed to the low concentration, and the degree of elongation was significant in the branches exposed to 0.05 mg/L (Fig. 1b). In contrast, there was no significant difference in the root length after seven days of exposure between the treatments.

The dry weight of the branches tended to decrease progressively with an increase in the Roundup concentration, and a significant difference was obtained between the plants treated with a concentration of 0.005 mg/L and the plants treated with 50 mg/L (Fig. 1c). Similarly, the chlorophyll-a contents tended to decrease with an increase in the concentration. The groups exposed to 5.0 and 50 mg/L presented significantly lower chlorophyll-a contents (except 0.5 mg/L) than the other groups (Fig. 1d).

Conclusion

In this work, branches were exposed to a wide range of Roundup concentrations in brackish water to mimic exposure via surface water after the application of this herbicide near littoral environments (high concentrations) or through runoff or leaching from terrestrial applications (low concentrations).

The binding to and blocking of EPSPS to inhibit the function of the shikimic acid pathway causes a deficiency in aromatic amino acids, leading to plant death due to starvation and increasing the plant's susceptibility to disease. This metabolic impairment likely result in mortality, prevents the formation of new leaves, and reduces the weight and chlorophyll-a contents in *R. maritima* branches.

Concentrations tested in this study stimulates changes in the leaf length of *R. maritima* and a slight increase in the dry weight. This increase in the leaf area increases the ability of the plant to equalize energy production through photosynthesis. These changes may suggest that the plants were stressed and therefore accessed compensatory mechanisms achieve homeostasis.

In general, the effects of a 7-day exposure on morphometric parameters were not significant or were only significant with extremely high concentrations (not normally found in the environment). Thus, specific biochemical, histological, and physiological assays may allow the identification of the short-term sub lethal effects exerted by exposure to low Roundup concentrations.

The highest concentrations of glyphosate affected the growth of *R. maritima*, as evidenced by a reduction in the weight differences of the leaves and a reduction in the content of chlorophyll *a*. These results indicate that contribution of this pollutant to aquatic environments can affect the development of *R. maritima* stands. Thus, there is an evident need for further warning against the use of herbicides near watercourse regions.

Assessment and conclusion

<u>Assessment and conclusion by applicant:</u> as stated in the literature review document submitted with the renewal dossier

5.4.1 case b) Relevant but supplementary information: This paper presents information on the effects of glyphosate on a saline tolerant species. However, there is no glyphosate exposure presented in the paper so it is very difficult to relate the observed effects to an exposure event / agricultural application.

Further point of clarification
The test item purity is not stated in the paper. The test substance was identified only as 'Roundup'. The surfactant system in the formulated product used in this study was not confirmed by the author. It is therefore not possible to confirm whether the product used, is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with high level of caution.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

<u>Assessment and conclusion by RMS:</u>
The aim of this study was to verify the acute effects of the formulated herbicide Roundup on the non-target species *R. maritima* under laboratory conditions (7 days exposure). No endpoint can be derived from the results as presented. No analytical verification was presented.
The study is less relevant but supplementary (formulation) and considered not reliable by RMS.

Data point:	CA 9
Report author	Smedbol E. *et al.*
Report year	2018
Report title	Effects of low concentrations of glyphosate-based herbicide factor 540® on an agricultural stream freshwater phytoplankton community.
Document No	Chemosphere (2018), Vol. 192, pp. 133-141
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

The effects of the exposure (96 h) of a phytoplankton community collected in an agricultural stream to various glyphosate concentrations (1, 5, 10, 50, 100, 500 and 1000 µg/L) of Factor 540® GBH were investigated. The lowest GBH concentration of 1 µg/L reduced chlorophyll *a* and carotenoid contents. Low glyphosate concentrations, such as 5 and 10 µg/L, promoted changes in the community's structure and reduced the diversity of the main algal species. At glyphosate concentrations ranging from 50 to 1000 µg/L, the phytoplankton community's composition was modified and new main species appeared. The highest glyphosate concentrations (500 and 1000 µg/L) affected the shikimate content, the lipid peroxidation and the activity of antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase). These results indicate that GBH can modify structural and functional properties of freshwater phytoplankton communities living in streams located in agricultural areas at glyphosate concentrations much inferior to the 800 µg/L threshold set by the Canadian guidelines for the protection of aquatic life.

Materials and methods

Sampling and test conditions

The phytoplankton community used in this study was collected from the Dumontier stream (45º36'41.38''N and 73º51'38.55'' W), located in southern Quebec (Canada), near the city of Boisbriand, a region characterized by field crop agriculture and pressure from urban sprawl. The agricultural site, adjacent to the sampled stream, was under GR soybean and GR maize rotation. The water sampling was performed in summer (2012-07-21), 42 days after the latest GBH application. The sampled water was filtered through nylon mesh (250 µm) to eliminate filamentous macroalgae and suspended macroparticles. To avoid nutritional constrain, soluble reactive phosphorus, inorganic nitrogen and silicate were added to the collected water following Berard (1996). After agitating for several minutes, the pH was checked and corrected to its original value (7.4). To remove the presence of zooplankton, the water was filtered a second time using a nylon mesh (40 µm), and then the filtrate (200 ml) was transferred into 500 ml Erlenmeyer flasks. A commercial GBH formulation, Factor 540® (IPCO, Winnipeg, Manitoba, Canada) was used in the present study. This GBH was the one used in the fields neighbouring the sampling site. Throughout this paper, the researchers refer to glyphosate concentrations of pure active substance present in the Factor 540® formulation. A pesticide stock solution of 54 mg/L concentration was prepared from the commercial formulation Factor 540®, in 0.22 µm filtered Dumontier stream water. After agitation, the solution was kept at 4ºC and was added directly into the Erlenmeyer flasks containing the phytoplankton samples (three replicates for each concentration). Glyphosate concentrations (0, 1, 5, 10, 50, 100, 500 and 1000 µg/L) were chosen based on the range of environmental concentrations found in agricultural water streams in Canada and the United States. Phytoplankton samples were then placed in an environmental growth chamber (MTR30, Conviron, Manitoba, Canada), under similar temperature and light conditions as the ones observed on the day before sampling (150 E m^{-2} s^{-1}; light dark cycle of 16:8 and average temperature of 15 °C, determined with a Hobo temp probe and data logger (Onset, Massachusetts, USA)), and agitated manually twice daily. Phytoplankton cell density was the same as the one found in the stream at sampling. The sampling was conducted after a period of 96 h of exposure to GBH. Glyphosate concentration originally present in the stream was evaluated at 1 g/L (± 0.02 g/L) using an enzyme linked immunosorbent assay (ELISA) (Abraxis LLC, Warminster, Pennsylvania, USA) with a detection limit of 0.05 µg/L.

Phytoplankton were identified and counted in all samples. Abundance (cells/mL) and per-cell biovolume (mm^3 ml^{-1}) were estimated based on number of cells, as well as cell measurements in length, width and thickness. The main species of the phytoplankton community were chosen as the species representing more than 5% of the total biomass. The photosynthetic activity was determined by measuring chlorophyll fluorescence. Shikimic acid concentrations were also evaluated.

Statistical analysis

Mean comparisons were conducted using the paired T-test, comparing the different treatments (1, 5, 10, 50, 100, 500 and 1000 µg/L) to the control treatment for growth indicators, pigment and shikimate

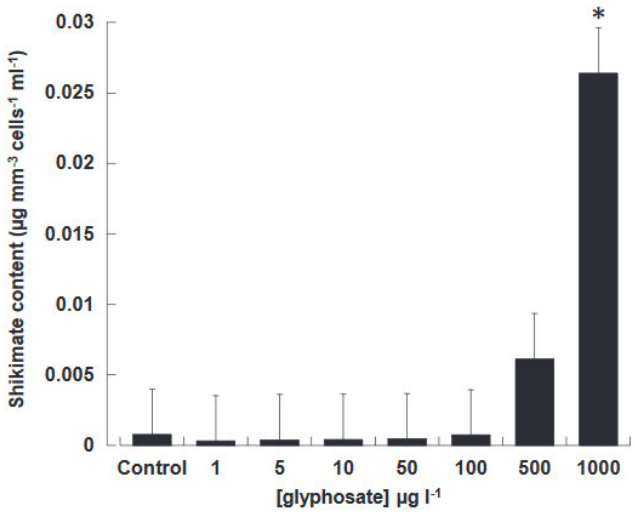
content, and enzymatic activity. Data were tested for normal distribution using the Shapiro-Wilk test, and for variances' homogeneity. All variables, except the dry weight one, did not fulfill these conditions and were therefore logarithmically transformed. All statistical analyses were performed by using JMP 12 software from SAS Institute.

Results

Shikimate content

The shikimate content showed a significant increase in phyto-plankton cells exposed only to 1000 µg/L of glyphosate present in the Factor 540® formulation (Fig. 1).

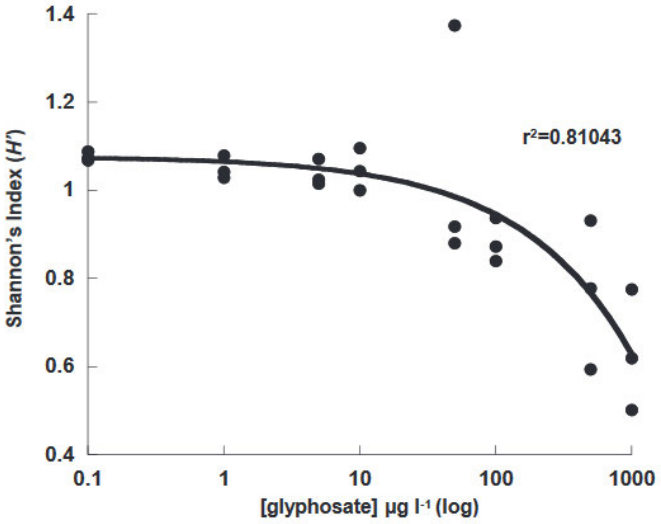
Figure 1. Shikimate content (µg mm^{-3} $cells^{-1}$ mL^{-1}) (mean and standard error) in an agricultural stream phytoplankton community after a 96 h' exposure to increasing glyphosate concentrations. The * symbol represents treatment with significant differences from the control treatment ($p<0.05$) by the paired T-test.



Diversity index

A fitted non-linear logistic regression (3 parameters) test between the Shannon diversity index and the glyphosate concentrations present in the Factor 540® formulation indicates that the diversity of the phytoplankton community sampled in the Dumontier stream, (Boisbriand, Quebec, Canada) decreased with the increasing glyphosate concentrations (Fig. 2).

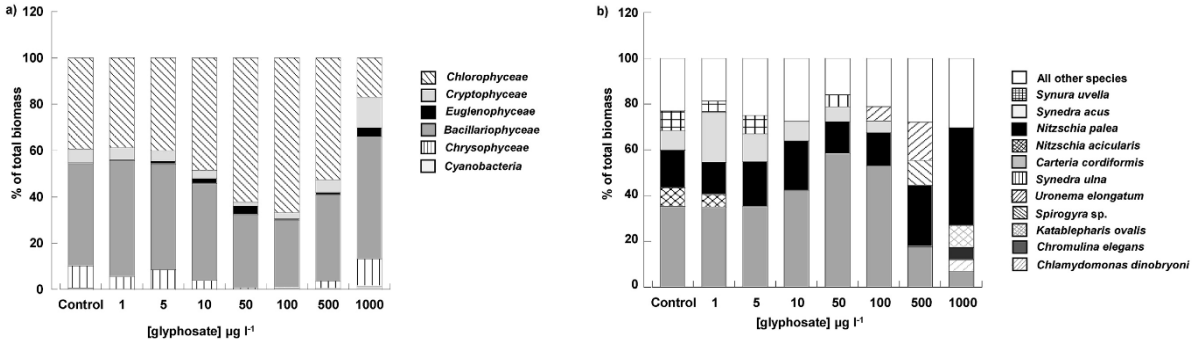
Figure 2. Relation between the Shannon's diversity index (H') and increasing glyphosate concentrations added to the growth media in an agricultural stream phytoplankton community after a 96 h exposure.



Interspecific variations

As shown in Fig. 3a, the phytoplankton community was composed of six main phytoplankton classes. The control treatment was dominated by the Chlorophyceae (40%), Bacillariophyceae (25%), Chrysophyceae (22%) and Chryptophyceae (10%). The presence of the Euglenophyceae (0.8%) and Cyanobacteria (2.2%) was marginal. When glyphosate concentrations present in the Factor 540® formulation increased (up to 100 µg/L), the relative biomass of the Chrysophyceae, Bacillariophyceae, Euglenophyceae and Cryptophyceae gradually decreased, while the relative biomass of the Chlorophyceae increased. At glyphosate concentrations, higher than 100 µg/L, the relative biomass of the Chlorophyceae started to decrease, while an increase was observed for the other four phytoplankton classes. At the highest glyphosate concentration (1000 µg/L), the phytoplankton community was composed of the Bacillariophyceae (35%), Chryptophyceae (30%), Chrysophyceae (14.5%), Chlorophyceae (12.7%), Euglenophyceae (5.6%) and Cyanobacteria (2%). Fig. 3b shows the shift in the composition of the phytoplankton species when exposed to different glyphosate concentrations for 96 h.We identified five main species for the control (*Carteria cordiformis* [35%], *Nitzchia palea* [16%], *Synedra uvella* [9%], *Nitzchia acicularis* [8%] and *Synedra acus* [8%]). For glyphosate concentrations, equal or above 5 µg/L, *N. acicularis* relative biomass decreased to below 5%. Similar results were obtained for *S. uvella* when exposed to glyphosate concentrations equal or above 10 µg/L. When glyphosate concentrations were 500 µg/L and higher, the proportion of *S. acus* in the community was also reduced to below 5% of total biomass. For glyphosate concentrations, equal or above 50 µg/L we noticed that new species emerged in the community as representative ones. Indeed, *Synedra ulna* was found at a relative biomass higher than 5% only when glyphosate concentration was 50 µg/L and *Uronema elongatum* was significantly present at 100 and 500 µg/L, and *Spirogyra sp*. was found to be a main species at 500 µg/L. The phytoplankton community observed at 1000 µg/L was quite different from that of the control treatment, with the diatom *N. palea* as the main species (43%) and with the emergence of three new main species: *Kateblepharis ovalis* (10%), *Chromulina elegans* (5.3%) and *Chlamydomonas dinobryoni* (5.1%). The proportion of the green alga *C. cordiformis* only started to decrease at glyphosate concentrations of 500 µg/L and above.

Figure 3. a) Relative biomass of the six major phytoplankton classes and b) Relative biomass of the major phytoplankton species (more than 5% of total biomass) in an agricultural stream phytoplankton community exposed 96 h to increasing glyphosate concentrations.



Photosynthetic parameters

The effective quantum yield and α were significantly reduced at respectively 500 and 1000 µg/L, and 1000 µg/L glyphosate concentrations present in the Factor 540® formulation compared to the control. The minimal saturating irradiance (EK) was reduced at various concentrations: 5, 10, 100 and 1000 µg/L. The relative maximal electron transport rate was the most sensitive parameter and was significantly reduce at all glyphosate concentrations (except for 50 µg/L) (Fig. 4).

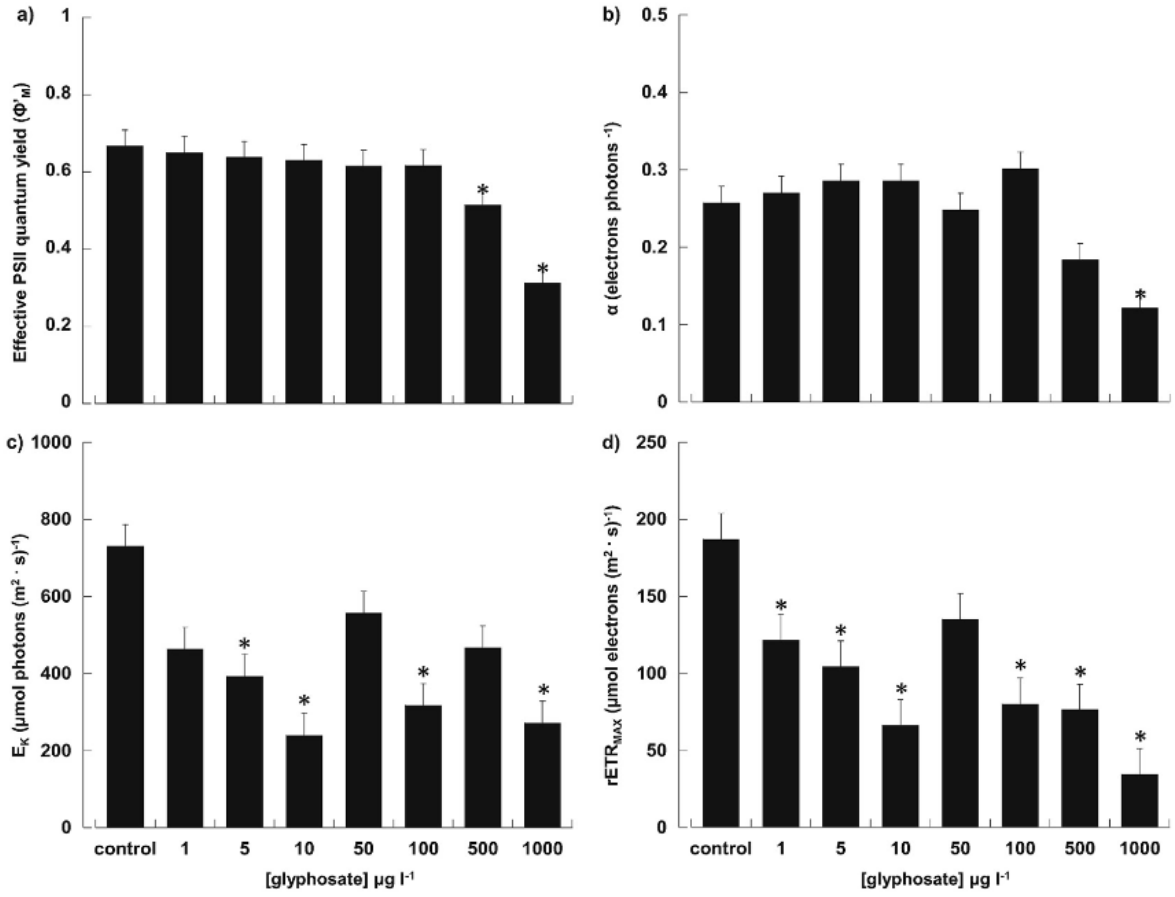
Growth indicators

Glyphosate's effects on the growth indicators (dry weight, species abundance and per-cell biovolume) of the phytoplankton community were evaluated (Table 1). Dry weight, species abundance and per-cell biovolume all decreased for the community exposed to high glyphosate concentrations present in the Factor 540® formulation (500 and 1000 µg/L). Treatments means and standard deviation (n = 3). The * symbol represents significant differences from the control treatment ($p < 0.05$) by the paired T-test.

Table 1. Growth indicators (dry weight [µg/mL], abundance [cells/mL], per-cell biovolume [mm^3/mL]), pigments content (chlorophyll a, chlorophyll b and carotenoid contents [% from control]) and chlorophyll a/b ratio in an agricultural stream phytoplankton community after a 96h exposure to increasing glyphosate concentrations.

	Glyphosate concentrations (µg l^{-1})							
	Control	1	5	10	50	100	500	1000
Dry weight	69.33 ± 14.7	76.7 ± 13.3	74.7 ± 16	84 ± 5.7	62.7 ± 4.2	73.3 ± 7	41* ± 4.2	34.6* ± 4.6
Abundance	2.3e+4 ± 1.5e+3	2.4e+4 ± 1.1e+3	2e+4 ± 1.2e+3	2.4e+4 ± 2.1e+3	1.6e+4* ± 1.6e+3	1.7e+4 ± 4.2e+3	1.1e+4* ± 3.2e+3	9.6e+3* ± 1.8e+3
Cell biovolume	3.3e-4 ±8.1e-4	4e-4 ±1.2e-3	4.4e-4 ±1.1e-3	3.9e-4 ±1.3e-3	4.3e-4 ±1.5e-3	3.3e-4 ±1e-3	8.1e-5* ±1.6e-4	3.3e-5* ±4.9e-5
Chl *a*	100 ± 0.8	61.7* ± 21	69.3* ± 13	94.7 ± 22	100.3 ± 18	66.1* ± 23	29.7* ± 29	28* ± 4
Chl *b*	100 ± 0.5	84.2 ± 42	83.8 ± 17	116.2 ± 17	134.3 ± 19	137.4 ± 96	100.6 ± 113	158.4 ± 79
Chl *a*/*b* ratio	2.46 ± 0.33	1.75* ± 0.44	1.88* ± 0.12	1.83* ± 0.20	1.69* ± 0.08	1.26* ± 0.42	0.73* ± 0.28	0.48* ± 0.25
Carotenoids	100 ± 5	32.9* ± 24	46.9* ± 18	56.2* ± 24	63.4* ± 10	27.4* ± 22	4.3* ± 5	1.1* ± 2

Figure 4. a) Effective PSII quantum yield b) Initial slope (α) c) Relative maximal electron transport rate ($rETR_{MAX}$) and d) Minimal saturating irradiance (EK) of the agricultural stream phytoplankton community exposed 96 h to increasing glyphosate concentrations, where the * symbol represents treatment with significant differences from the control treatment ($p<0.05$) by the paired T-test.



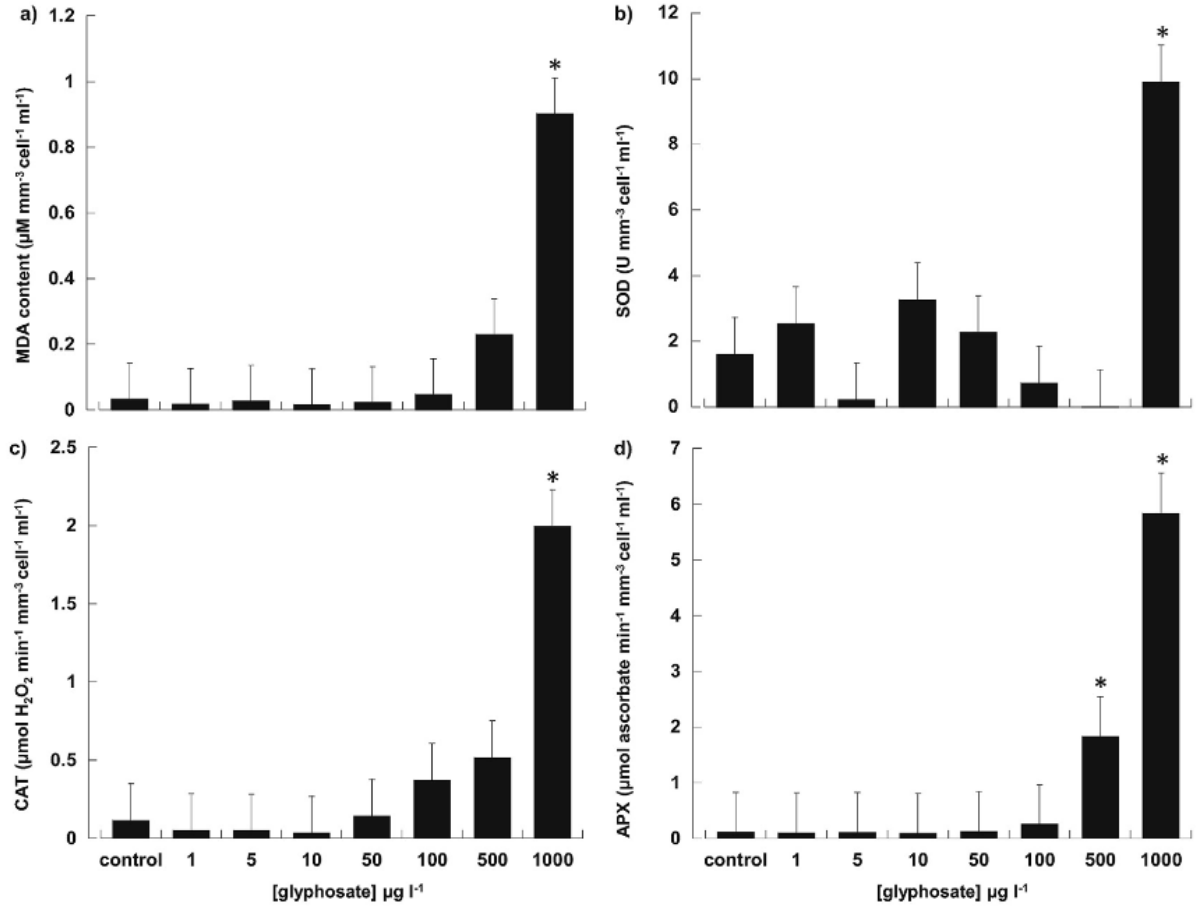
Pigment content

Chlorophyll *a* and carotenoid content (% of pigment compared to control) significantly decreased in phytoplankton cells for all glyphosate concentrations, while there was no significant difference for chlorophyll *b* content (Table 1). The chlorophyll *a/b* ratio decreased following the increasing glyphosate concentrations.

Oxidative marker

Lipid peroxidation (MDA concentration), SOD and CAT activities were significantly increased in samples exposed to the highest glyphosate concentration, when compared to the control, while APX activity increased for samples treated with glyphosate concentrations of 500 and 1000 µg/L(Fig. 5).

Figure 5. Oxidative stress markers (MDA content, superoxide dismutase [SOD], catalase [CAT] and ascorbate peroxidase [APX]) (mean and standard error) in an agricultural stream phytoplankton community after a 96 h exposure to increasing glyphosate concentrations, where the * symbol represents treatment with significant differences from the control treatment ($p<0.05$) by the paired T-test.



Conclusion

Glyphosate negatively affected the freshwater phytoplankton community of the studied agricultural stream. The results indicate that GBH Factor 540® can affect the structure of the phytoplankton community at low glyphosate concentrations which are characteristic of the environmental concentrations monitored by different studies. The observed short-term effects of low glyphosate concentrations present in the Factor 540® formulation on freshwater phytoplankton communities questions the effectiveness of the acute toxicity standard for aquatic life (27 000 µg/L of glyphosate; CCME, 2012) used for managing GBH risks. Lastly, the study highlights the hypothesis that the glyphosate's toxic effects on phytoplankton may be linked to a perturbation of the photosynthetic apparatus possibly mediated through oxidative stress.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Studies which can be difficult to extrapolate to EU (e.g. with local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). In addition, the tested formulation Factor 540®, is not the representative formulation for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

Factor 540® was used. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. The study is considered less relevant but supplementary (formulation issue).

Effects of the exposure (96 h) of a phytoplankton community collected in an agricultural stream to various glyphosate concentrations (1, 5, 10, 50, 100, 500 and 1000 µg/L*) of Factor 540® GBH were investigated.
Typing errors are noted (concentrations are wrongly expressed as g/L instead of µg/L in the text).

- The lowest GBH concentration of 1 µg/L reduced chlorophyll a and carotenoid contents.
- Low glyphosate concentrations, such as 5 and 10 µg/L, promoted changes in the community's structure and reduced the diversity of the main algal species.
- From 50 to 1000 µg/L, the phytoplankton community's composition was modified and new main species appeared.
- The highest glyphosate concentrations (500 and 1000 µg/L) affected the shikimate content, the lipid peroxidation and the activity of antioxidant enzymes (superoxide dismutase, catalase and ascorbate peroxidase).

This study suggests that Factor 540® GBH can modify structural and functional properties of freshwater phytoplankton communities living in streams located in agricultural areas at low glyphosate concentrations.

No analytical verification was made.
There is no information on environmental conditions (e.g. oxygen, nutrient levels). The variation in the composition in the community between replicates is not shown which makes it difficult to assess the magnitude of the effect. It is not known how the communities look like at the start of the study (sampling at 96h only). There is only a comparison with the 96h-changed control treatment. A sample at t=0 is missing. It is also noted that glyphosate concentration originally present in the stream was evaluated at 1 g/L (±0.02 g/L). While assuming the unit is wrongly expressed in g/L, the presence of glyphosate renders the outcome of the study doubtful.

RMS considers that this study is less relevant but supplementary (different formulation tested) and not reliable.

6. Other aquatic organisms

Data point:	CA 8.2
Report author	Li Jiao et al.
Report year	2010
Report title	Acute Toxicity of Eight Pesticides on the Development of Sea Urchin Embryos.
Document Source	Asian Journal of Ecotoxicology (2010), Vol. 5, No. 2, pp. 255
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Acute toxic effects of glyphosate concentrations of 0.1, 0.5, 0.8, 1, 4, 6, 8, 10, 20, 30, and 50 mg glyphosate/L on the embryo development of the sea urchin *Strongylocentrotus intermedius* were evaluated. Toxicity was quantified in terms of the EC_{50} (median effective concentration) on the six early developmental stages of sea urchin embryo (2-cell, 4-cell, blastula, gastrula, prism, and 4-arm pluteus). In addition, the concentration-response curves for each pesticide and the relationship between octanol/water partition coefficient (logP) were analyzed.
Acute toxicity of glyphosate causes gradually decrease of the EC_{50} with the development of the embryo with a lowest EC_{50} of 3.99 mg/L at the last stage, the 4-arm pluteus.

Materials and methods

Material
Strongylocentyotus internedius used for the test was taken from the key laboratory of Dalian Ocean University, with a shell diameter of 3.6 - 4.8 cm and a shell height of 2.2 - 3.1 cm. The seawater used in the test was taken from the sea near Blackstone Reef in Dalian. The salinity was 32.2 ± 0.3 and pH was 8.13 ± 0.5.
Glyphosate was purchased from the Institute for the Control of Pharmaceutical and Biological Products of China. Glyphosate was dissolved in fresh seawater. The pre-test was carried out according to the acute toxicity test method to determine the dose range of the development of sea urchin embryo from fertilization to the total death and no death of four-wrist larvae, and the experimental concentration was set according to the pre-experiment: 0.1, 0.5, 0.8, 1, 4, 6, 8, 10, 20, 30, and 50 mg glyphosate/L. The test contained 3 parallel concentrations, 1 control group and 1 control group for filtering seawater.

Toxicity tests
After fertilization, the egg was washed twice and developed in a 250 mL beaker containing 200 mL of experimental solution. The density of the fertilized egg was approximately 100-150 g/mL. The embryo

development process was observed. In the course of the experiment, six developmental stages were selected for observation: two-cell stage, four-cell stage, upper-vesicle embryo stage, gastrula stage, prismatic larva stage and four-wrist larva stage. After fertilization, the control group was observed to ensure that more than 90% of the eggs were regularly fertilized. In this experiment, it took 1.5 h for the control group to develop to two cells, 2.5 h for the development to four cells, 16.5 h for the development to the upper floating blastocyst, 28.5 h for the development to the gastrula stage, 41 h for the development to the prismatic larvae, and 52 h for the development to the four wrist larvae. When observed, each parallel random sampling of about 100 individuals were fixed with 5% formaldehyde and counted. Normal individuals entering all periods of development are counted as normal individuals, and individuals with developmental delays, deformities and deaths are counted as inhibited individuals. The experimental process is intermediately inflated to ensure that sufficient oxygen enters the experimental water body, and the water temperature is controlled at 18 °C.

The Calculation of EC_{50} Value
The inhibition rate of sea urchin embryo development in each experimental group was examined and counted. Using SPSS 13.0 software to calculate the EC_{50} value of pesticide for each development period of sea urchin embryos.

The correlation analysis of LogP and EC_{50}
Chmeoffice software was used to calculate the LogP value of 8 pesticides, and SPSS 13.0 software was used to make graphics and analysis for the correlation of LogP and EC_{50}.

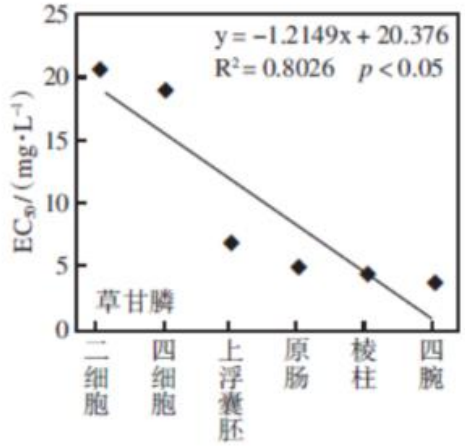
Results

Acute toxicity of glyphosate to each stage of development of sea urchin embryos
The EC_{50} gradually decreased with the development of the embryo and the toxicity of pesticides to the embryo gradually increased, indicating that with the development of sea urchin embryos, its sensitivity to pesticides was enhanced, among which the four-wrist larval stage was the most sensitive and the tolerance was the lowest (Table 1 and Fig. 1).

Table 1: EC_{50} values of glyphosate for the development of sea urchin embryos

Development period	**2-cell**	**4-cell**	**Upper floating**	**Gastrula stage**	**Prismatic larvae**	**4-arm larvae**
EC_{50} [mg/L]	20.52 (16.40 – 25.38)	19.02 (16.22 – 22.26)	7.03 (4.98 – 9.28)	5.02 (3.08 – 6.20)	4.72 (3.93 – 5.35)	3.99 (2.89 – 4.61)

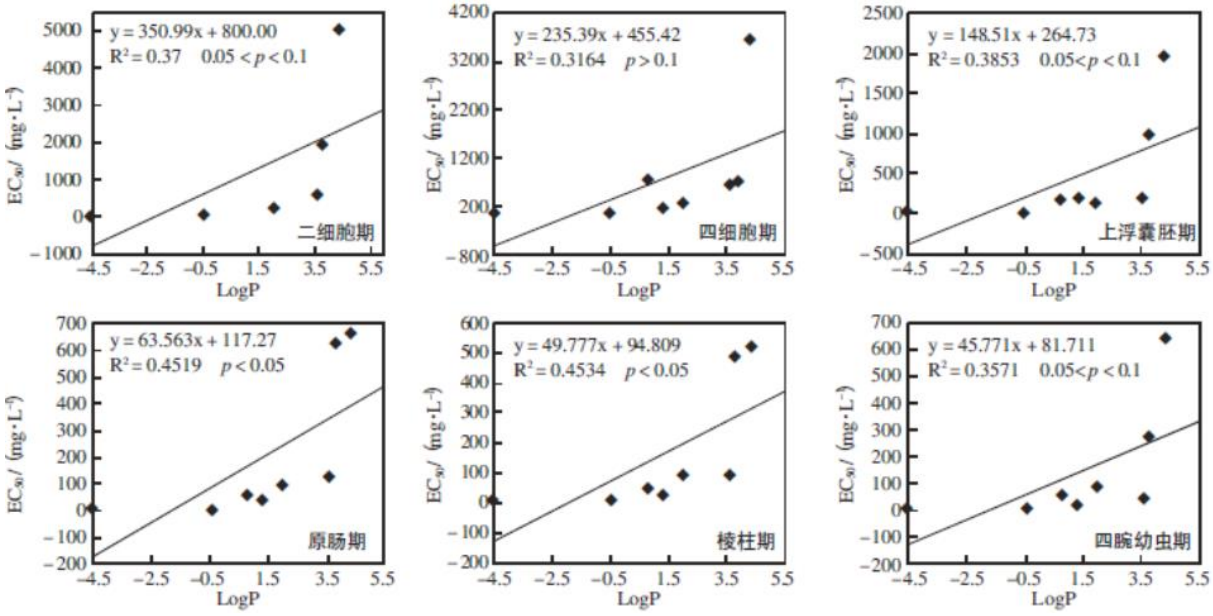
Figure 1: Relationship between EC_{50} values and sea urchin embryo development periods at glyphosate exposure. *(Note: from left to right under the line: 2-cell, 4-cell, Upper floating, Gastrula stage, Prismatic larvae, 4-arm larvae).*



The correlation analysis of LogP and EC_{50}

The relationship between LogP and EC_{50} was plotted using the LogP value of glyphosate as the horizontal coordinate and the EC_{50} value of each embryo of sea urchin as the vertical coordinate. The increase of pesticide LogP, each pesticide showed a gradual upward trend towards the EC_{50} of embryos of different developmental stages. Regression analysis showed that the EC_{50} in the gastrula stage and the prismatic larvae stage had a good positive correlation with logP ($p < 0.05$) (Fig. 2).

Figure 2: Relationship between LogP values and EC_{50} values during different sea urchin embryo development periods.



Conclusion

Acute toxicity of glyphosate causes gradually decrease of the EC_{50} with the development of the sea urchin (*Strongylocentrotus intermedius)* embryo with a lowest EC_{50} of 3.99 mg/L at the last stage, the 4-arm pluteus (52 h development).

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study of the toxicity to the sea urchin embryos, was not conducted or based on a relevant guideline. Test concentrations were from 0.1 to 50 mg/L of glyphosate technical. The relationship between EC_{50} and LogP values was the main discussion of the article In addition, it is a non-EU study conducted with local native species and influenced by different geo-climatic properties and land-uses and agricultural practices. The study does not present any data which can be used in the ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Assessment and conclusion by RMS:

Original report in Chinese, results are based on a document translated in english.
Acute toxic effects of glyphosate on the embryo development of the sea urchin Strongylocentrotus intermedius were investigated.

Acute toxicity of glyphosate causes gradually decrease of the EC50 with the development of the sea urchin (Strongylocentrotus intermedius) embryo with a lowest EC50 of 3.99 mg/L at the last stage, the 4-arm pluteus (52 h development).

It was not based on a relevant guideline (no guideline available). Only EC50 is available, no biological data were presented. No NOEC was derived.

RMS considers that this study is of low relevance as it does not present any data which can be used in the ecotoxicological regulatory risk assessment.

Less relevant but supplementary (endpoint not relatable to the risk assessment scheme) and not reliable (no data available in the report).

Data point:	CA 8.2
Report author	Puértolas L. et al.
Report year	2010
Report title	Evaluation of side-effects of glyphosate mediated control of giant reed (*Arundo donax*) on the structure and function of a nearby Mediterranean river ecosystem.
Document Source	Environmental research (2010), Vol. 110, No. 6, pp. 556-564
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

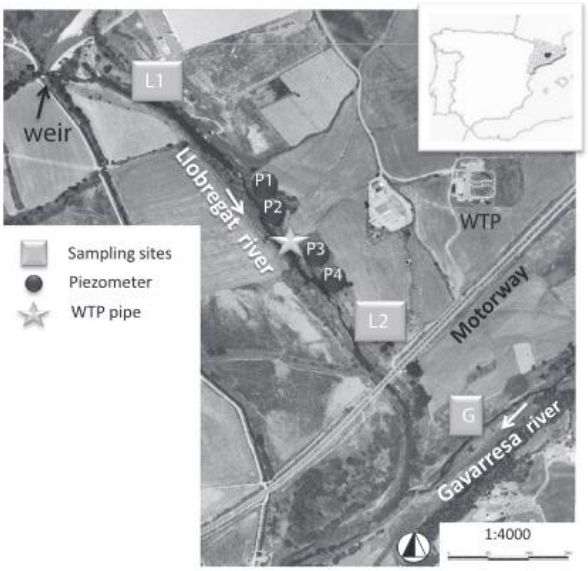
The aim of this study was to evaluate the effect of the application of the glyphosate-based herbicide Herbolex on the structure and function of a nearby river ecosystem after application at 2.1 kg glyphosate/ha in an area of 0.5 ha of riparian forest. Therefore, the glyphosate environmental fate in the surrounding water and its effects on transplanted *Daphnia magna*, field collected caddisfly (*Hydropsyche exocellata*) and on benthic macroinvertebrate structure assemblages were assessed. Investigations were conducted in the industrialized and urbanized Mediterranean river Llobregat (NE Spain) before and after a terrestrial spray of glyphosate. Four locations were selected to include an upstream site and three affected ones.
Measured glyphosate levels in river water were quite high (20–60 µg/L) with peak values of 137 µg/L after three days. After 12 days of its application, leaching of glyphosate from sprayed riverbanks was quite high in pore water (20–85 µg/l) but not in the river. Closely linked with the measured poor habitat and water physico-chemical conditions, macroinvertebrate communities were dominated by taxa tolerant to pollution and herbicide application did not affect the abundance or number of taxa in any location. Nevertheless, significant specific toxic effects on transplanted *D. magna* and field collected *H. exocellata* were observed. Effects included *D. magna* feeding inhibition.

Materials and methods

Test item
Herbolex is a mixture containing a concentration of glyphosate isopropylamine salts of 486 and 200 g/L of surfactant compounds.

Study site and sampling dates
The study was conducted in the Llobregat river basin (Catalonia, NE Spain), which supply water to the city of Barcelona and is a good example of an intensively used Mediterranean stream system, being impacted by urban, agricultural and industrial activities. On behalf of river restoration project to control the giant reed, glyphosate was applied in the riparian vegetation across a restricted area in the mid section of the Llobregat river basin (Fig. 1).

Figure 1. Study site in the Llobregat and Gavarresa junction in Catalonia (NE, Spain). Boxes are sampling sites: L1, L2 and G. Piezometer stations in spots from P1 to P4, two located upstream of the water treatment plant (WTP) pipe and two downstream.



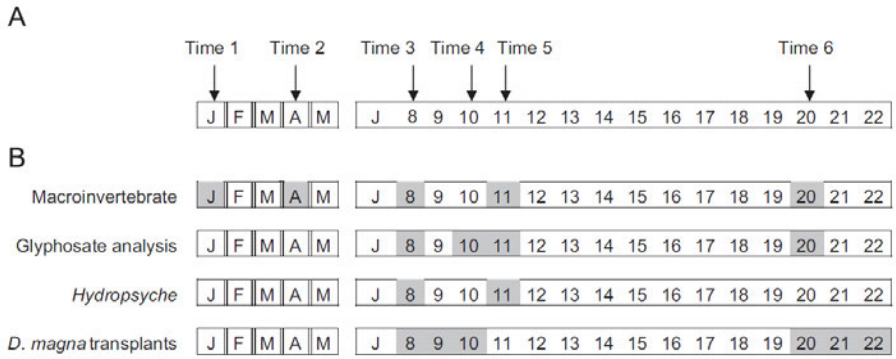
The side-effects of this application were evaluated at three locations: L1 and L2 situated in the river Llobregat, up and downstream of a small sewage outflow, respectively, while a third location was placed in the Gavarresa stream (G), prior to its confluence with the Llobregat main channel. Llobregat is a middle mountain Mediterranean river type with a relatively high discharge while Gavarresa river type is lowland Mediterranean with more variable and lower discharge.

The selected three sites are characterized by showing a moderate ecological quality due to degraded riparian vegetation and poor water quality, specially due to salt discharge from an upstream mine.

Field application
Terrestrial application of herbicide (2.1 kg glyphosate/ha) was conducted in an area of 0.5 ha of riparian forest by the enterprise on June 8th, 2007, at these locations. Three months before spraying of leafs, stems were cut and reeds sprouted up to 1 m high.

Field monitoring
The monitoring program included 6 samplings: time 1,5 months before pesticide application on 13th January 2007; time 2, two months before, on 24th April 2007; time 3, just on herbicide application, on 8th June 2007; and two, three and 12 days after (time 4, 5 and 6).
Both glyphosate and its major metabolite AMPA were determined from river water samples collected from the studied sites at times 3, 4, 5 and 6. In addition, contaminant levels in the pore water of the sprayed riverbanks were also measured using four piezometers deployed across the studied riverbank site (Fig. 1). PVC piezometers were 1 m long, completely porous and water extraction was made by manual suction.
The structure of the benthic macroinvertebrate assemblages was assessed at times 1, 2, 3, 5 and 6. Transplants with *Daphnia magna* were deployed at times 3 and 6, whereas *Hydropsyche exocellata* samples were collected at times 3 and 5. The previous sampling schedule, summarized in Fig. 2, was selected to increase recent historical data of the studied communities before treatment (time 1-2), and to include the periods of exposure to (time 3-5) and post-exposure (time 6) to the herbicide. Due to experimental constrains it was not possible to deploy *D. magna* organism just prior to herbicide application; thus transplants conducted at time 6 can be considered as no exposure to the herbicide.

Figure 2. Sampling scheme diagram. Months of sampling periods, time 1-6, and the exact days of sampling in June are depicted (A). Gray boxes indicate the sampling periods used for macroinvertebrates, glyphosate analyses, *Hydropsyche* samples and *Daphnia magna* transplants (B). J, F, M, A, M and J are January, February, March, April, May and June, respectively.



Environmental measurements

A set of environmental variables was measured on each sampling or deployment period. Discharge was determined using a Mini Air flow meter and making a cross river section (transect) and measuring water flow at different depths across it. Water physico-chemical parameters, such as temperature, pH, conductivity and dissolved oxygen, were measured in situ by using a WTW Multi 340i handheld meter, whereas total suspended solids, TOC, DOC, anions, cations, NH_4, NO_2, NO_3, PO_4, SO_4 and Cl were measured in the lab following ASTM Standard Methods (ASTM 1995). An additional 500 ml of water was stored in clean amber bottles at 4 °C until analysis.

Chemical analysis

Chemical analyses were restricted to glyphosate and its major metabolite aminomethylphosphonic acid (AMPA). A standardized method was adopted to the specific characteristics of the two environmental water matrices (Llobregat and Gavarresa) that showed a relatively high conductivity due to the presence of salt.

Half a liter of water was concentrated to 1 mL using a rotor evaporator. To remove precipitated salts water extracts were treated with ethyl acetate at 50 °C and hot filtered with a hydrophobic filter. Once ethyl acetate was evaporated, samples were derivatized using trifluoroacetic anhydride and trifluoroethanol at 95 °C during 30 min. After the reaction, excess of reactives were evaporated and 400 mL of ethyl acetate was added prior to its analysis with a gas chromatograph coupled to a mass spectrometer from Shimadzu (Japan) model GCMS-QP2010. The mass spectrometer was operated in the negative chemical ionization mode. Compound separation was achieved using a capillary column VF-5 MS of 30 m × 0.25 mm i.d. with 0.25 mm film thickness from Varian Inc..

Identification and internal standard ion quantification were carried out automatically by the GCMS solutions software in version 2.5. Quality assurance included three concurrent replicate samples at each sampling day and location, the use of blanks (only ethyl acetate) and standard reference materials (SRM's). Both blanks and SRM's were prepared and analyzed within each batch of samples, both with pure water and river water matrices. SMR's included glyphosate at 98.0 % and AMPA 99.0 % at 0.1, 1, 10 and 100 µg/L.

Biological conditions
Biological responses focused on functional traits (in situ post-exposure feeding *D. magna*, sensu, specific responses (biomarkers) and community level effects (changes in benthic macroinvertebrate assemblages)). Benthic macroinvertebrates, riparian vegetation and habitat quality at the studied sites were studied in order to establish the ecological status of the sites using the Guadalmed protocol and to assess the extent to which benthic macroinvertebrate assemblages were affected by herbicide treatment. Benthic macroinvertebrates were obtained quantitatively by sampling all available habitats with a kick net of 250 mm during 8 min, specimens were then preserved in formalin (5%), identified to the family level and used to determine the biological quality of water (Prat and Munné, 2000; Prat et al., 2002).

In situ bioassays

Exposure regime
In situ *D. magna* deployments were conducted. In each deployment, a lab control treatment with animals maintained in the lab and never exposed to the field was also included as a surrogate control. Briefly the procedure for the in situ bioassays was as follows. Four day old juveniles were transported to field sites in groups of 10 in 175 glass jars filled with American Society for Testing Materials (ASTM) hard water. At each site 5-7 chambers, each containing 10 individuals, and 4 chambers containing 20 individuals, were placed inside a 13 mm^2 wire-mesh cylinder that was positioned in the stream perpendicular to flow.

Post-exposure responses
After 48 h, animals were retrieved from the chambers. Surviving animals from those chambers holding groups of 20 individuals were pooled in an Eppendorf and immediately frozen in liquid N_2 and kept at -80°C until further enzyme analysis.
Shortly after exposure (within 1 h) five surviving juveniles from those chambers holding groups of 10 animals were placed into 60 ml screw-capped glass jars containing 50 mL of ASTM hard water, with *Chlorella vulgaris* (Beijerink, strain CCAP C2 11/12) at a concentration of 5×10^5 cells/ml, and allowed to feed for 4 hours. Three jars containing no animals were used to establish initial algal densities. Biomarker and post-exposure feeding rates were also measured in animals maintained in the lab during the deployments and transported to the field sites to include a surrogate lab control. Post-feeding experiments were conducted in darkness to avoid algal growth and under constant temperature conditions (20 ±2°C) provided by a thermostatized chamber. Individual feeding rates (cells/animal×h) were determined as the change in cell density during 4 h. Cell density was estimated from absorbance measurements at $\lambda = 650$ nm in a dual-beam spectrophotometer (Uvikon 941) using standard calibration curves based on at least 20 data points, with an $r^2 > 0.98$.

Data analysis
Basic statistical analysis was performed with environmental and herbicide data with mean and standard error calculation. The structure and composition of benthic macroinvertebrate assemblages were characterized using a broad range of metrics adapted to river types and generated with the MAQBIR software. *D. magna* and *H. exocellata* responses across sites and deployments or sampling dates were compared by two way ANOVA followed by post-hoc Tuckey's multiple comparison test. Within each deployment Daphnia responses were transformed to proportions relative to the lab controls to account for inter-trail differences in the studied parameters. Prior to analyses *H. exocellata* and *D. magna* data was log and arcsine transformed, respectively, to meet ANOVA assumptions of normality and variance homoscedasticity.

Results

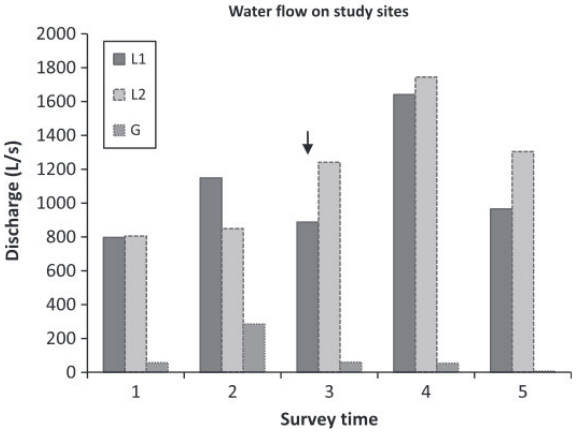
Physico-chemical water characteristics

In general, the area has not very good conditions for invertebrate fauna: relatively low water flows, high temperatures and conductivity of water and high nutrient content (Table 1). Water discharge is variable (Fig. 3), and it shows the effects of water extraction and flow regulation due to dams and weirs upstream of the study site for electricity generation purposes. Later in summer, the Gavarresa system tends to be more similar to a pond with low flow conditions, high conductivity values or high oxygen during daytime because of the amount of Cladophora and other algae present in the stream. In addition, conductivity values rarely decrease from 1000 µS/cm in both rivers and sometimes even reach values of more than 3000 µS/cm. This and other chemical parameters are presented in Table 1.

Table 1. Mean chemical composition (± SE) of river water on study sites during the sampling period (n = 5).

Parameter	L1	L2	G
Ox (mg/L)	9.1 (±0.9)	9.1(±1.1)	12.8(±1.3)
Cond (µS/cm)	1504.0 (±174.6)	1699.4(±199.7)	3001.2(±294.1)
Cl^- (ppm)	379.1(±34.4)	398.5(±43.0)	672.8(±124.5)
SO_4^- (ppm)	135.0(±18.0)	130.4(±15.0)	596.3(±90.7)
NO_3^- (ppm)	1.8(±0.5)	3.1(±0.1)	4.1(±2.2)
PO_4^{3-} (ppm)	0.3(±0.1)	0.4(±0.1)	0.6(±0.1)
TOC (ppm)	2.3(±0.4)	3.3(±0.8)	6.2(±1.8)
DOC (ppm)	3.4(±1.2)	3.2(±0.7)	4.1(±0.7)

Figure 3. Discharge evolution on the study sites between January and June 2007. The black arrow indicates herbicide treatment.



The presence of a sewage discharge coming from a water treatment plant (WTP) poses some differences among stations L1 and L2, especially in conductivity and nutrient parameters although no temporal trend is distinguishable in these sites.

Detection of glyphosate in river water

The standard curves for glyphosate for the Llobregat and Gavarresa rivers had a correlation (R^2) of 0.977 and 0.999, respectively. The limit of detection in the studied rivers for glyphosate and AMPA was 3 µg/L. Levels of glyphosate in surface and pore water are depicted in Table 2.

Table 2. Glyphosate (mean concentration with standard error) and AMPA for study samples.

Station and sampling scheme	Glyphosate (µg/l)	AMPA (µg/l)
Time 3		
L1	21.4 (±0.9)	<3
L2	31.0 (±0.2)	<3
G	3.6 (±0.0)	<3
P1	<3	<3
P2	<3	<3
P3	<3	<3
P4	<3	<3
Time 4		
L1	55.0 (±10.9)	<3
G	7.9 (±0.3)	<3
Time 5		
L1	40.6 (±26.8)	<3
L2	11.1 (±5.8)	<3
G	139.6 (±27.9)	<3
P1	<3	<3
P2	<3	<3
P3	<3	<3
P4	<3	<3
Time 6		
L1	<3	<3
L2	<3	<3
G	<3	<3
P1	<3	<3
P2	<3	<3
P3	89.8	<3
P4	26.8	<3

Chemical analyses of water only evidenced the presence of Glyphosate with no traces of AMPA. Glyphosate concentrations in surface water were only detected during application (time 3), two (time 4) and three days after (time 5) reaching a maximum of 137 µg/l at G station, levels decrease to 20-60 µg/Lin the Llobregat river channel after 3 days of application, and finally undetected levels were found on day 12. In the riverbank pore water, glyphosate levels were undetected until day 12 (time 6), when in piezometers situated farther away from the river reached levels of 28-89 µg/l.

Invertebrate community composition

Herbicide application did not affect the abundance or number of taxa of macroinvertebrates in any location (Fig. 4). Closely linked with poor chemical conditions, macroinvertebrate communities were dominated by taxa tolerant to pollution (Fig. 5).

Figure 4. Evolution of the macroinvertebrate abundance during the sampling period. The black arrow indicates the beginning of herbicide treatment.

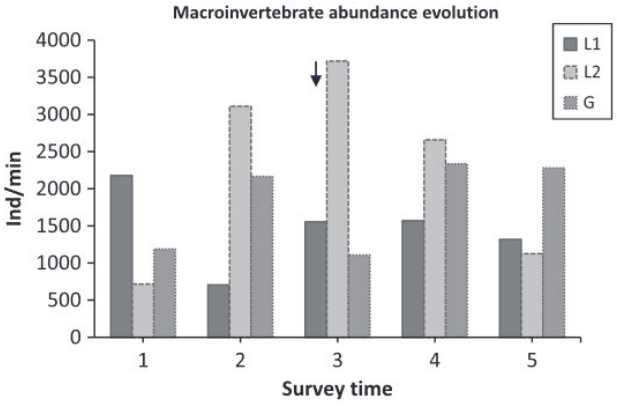
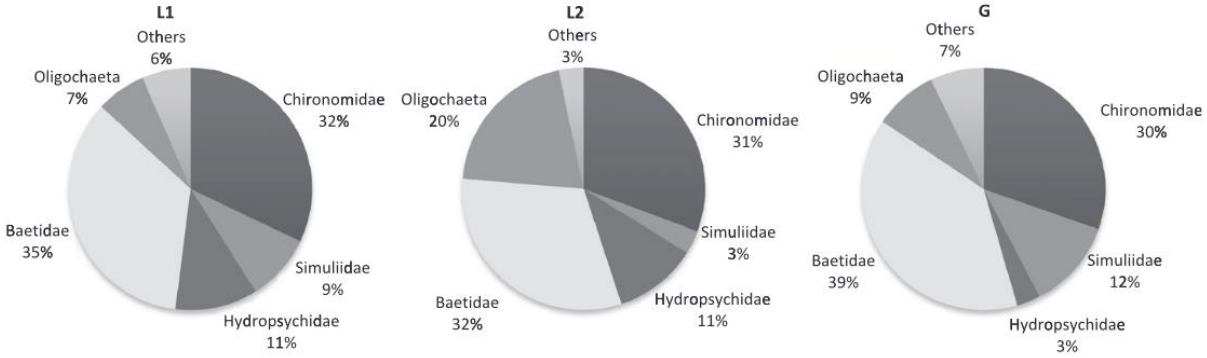


Figure 5. Mean proportion of the most common macroinvertebrate taxa at the study sites from January to June 2007.



Provided the small differences in water quality, the studied communities were quite similar among all the studied sites. One-third of the individuals belonged to Baetidae (*Ephemeroptera*) and Chironomidae (*Diptera*) families. Oligochaeta was also abundant, especially downstream of sewage discharge. The most abundant *Trichoptera* family was *Hydropsychidae*, and most of them were from the species *H. exocellata*.

Conclusion

The study aimed to evaluate the effect of the application of the glyphosate-based herbicide Herbolex, once applied to control giant reed at 2.1 kg glyphosate/ha, on the structure and function of a nearby river ecosystem in the industrialized and urbanized Mediterranean river Llobregat (NE Spain). Fate in the surrounding water on transplanted *Daphnia magna*, field collected *Hydropsyche exocellata* and on benthic macroinvertebrate was assessed. Investigations were conducted before and after a terrestrial spray of glyphosate.
Due to adopted microflora and exposure to glyphosate, macroinvertebrate communities were dominated by taxa tolerant to pollution and herbicide application. Neither abundance nor number of taxa in any location were affected but significant specific toxic effects on transplanted *D. magna* and field collected *H. exocellata* were observed. Effects on *D. magna* was feeding inhibition.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The effect of the herbicide Herbolex (mixture of glyphosate isopropylamine salts and surfactant compounds) on the structure and function of a nearby river ecosystem after application of glyphosate in the riparian vegetation was evaluated. Therefore, in situ bioassays with transplanted *Daphnia magna*, field collected caddis fly (*Hydropsyche exocellata*) and benthic macroinvertebrate structure and function were investigated. The structure of the benthic macroinvertebrate assemblages was assessed at the same time as well as two additional time-points before application (5 and two month before). Transplants with *Daphnia magna* were deployed at the day of application and 12 days afterwards, whereas *Hydropsyche exocellata* samples were collected at the day of application and 3 days afterwards. Concentration of glyphosate and the metabolite AMPA was analysed in the river water samples collected from the studied sites at the day of application and two, three and 12 days afterwards. But other chemicals were not analysed. The herbicide was applied at 2.1 kg glyphosate/ha in an area of 0.5 ha of riparian forest, but the exact place is not specified. Furthermore, no data on the weather conditions were collected which may have had an influence on the community structure. No exact biological data regarding the macroinvertebrate abundance is reported. However, as no results were reported in values reflecting agreed endpoints for the ecological risk assessment and the information is insufficient to transfer values in such endpoints.

In addition, a formulation was tested which is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The effect of the herbicide Herbolex (mixture of glyphosate isopropylamine salts and surfactant compounds) on the structure and function of a nearby river ecosystem after application of glyphosate in the riparian vegetation was evaluated. In situ bioassays with transplanted Daphnia magna, field collected caddis fly (Hydropsyche exocellata) and benthic macroinvertebrate structure and function were investigated.

The herbicide was applied at 2.1 kg glyphosate/ha in an area of 0.5 ha of riparian forest.

Transplants with Daphnia magna were deployed at the day of application and 12 days afterwards, whereas Hydropsyche exocellata samples were collected at the day of application and 3 days afterwards. Concentration of glyphosate and the metabolite AMPA was analysed in the river water samples collected from the studied sites at the day of application and two, three and 12 days afterwards.

No exact biological data regarding the macroinvertebrate abundance is reported. No effect was observed but it may be explained by a less diverse community dominated by tolerant species (according to study authors).

Significant specific toxic effects on transplanted D. magna and field collected H. exocellata were observed. Measured glyphosate concentrations ranging between 20 and 137 µg/l in water affected D.magna feeding rates and the activity of biotransformation (GST, CbE), antioxidant (GPX), metabolic (LDH) and/or anticholinergic enzymes of the two studied invertebrate species.

In each in situ D. magna deployments, a lab control treatment with animals maintained in the lab and never exposed to the field was included as a surrogate control.

The authors suggested interactive combined effects of naturally occurring factors and the herbicide application. Indeed the studied sites were characterized by difference in organic pollution (L2 received the sewage of a nearby small industrial park) and salinization (G was highly impacted by an excess of Cl and SO4). An excess of ammonium coming from water treatment plant, salts and changes in oxygen levels affected the studied behavioral and biochemical responses. Overall, due to poor water quality, RMS would not consider the results reliable.

RMS questions the comparison using lab controls (no in situ control was available). Effects may also be due to any other parameters related to the study site.
The study is less relevant but supplementary (due to different formulation tested) and not reliable.

Data point:	CA 8.2.7
Report author	Rzymski P. et al.
Report year	2013
Report title	The effect of glyphosate-based herbicide on aquatic organisms - a case study.

Document Source	Limnological Review (2013), Vol. 13, No. 4, pp. 215
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The deliberate use of glyphosate-based herbicide in a bathing area of Lake Lednica (Wielkopolska, Poland) by unknown perpetrators in April 2011 was described. The use of glyphosate was determined by chemical analysis of water and biological samples. The observed effects of this herbicide on emerged plants as well as the benthic macroinvertebrate community which inhabited associated sediments were reported.
Glyphosate was detected using gas chromatography mass spectrometry (GC-MS) in the water samples collected from the bathing area at a mean concentration of 0.09 mg/dm^3. Above ground parts of emerged macrophytes (*Phragmites australis* and *Typha latifolia*) covering the investigated area were completely withered. Studies of benthic macroinvertebrates revealed no significant differences in taxa number between event (13 taxa) and control (14 taxa) sites although differences in abundance of particular taxa were observed. Significantly lower numbers of *Chironomidae* (by 41 %), Oligochaeta (by 43 %), Viviparіae (by 75 %), Hirudinae (by 75%), *Asellus aquaticus* (by 77 %), *Gammarus pulex* (by 38 %) and *Dreissena polymorpha* (by 42 %) were found at the glyphosate-treated site. Furthermore, compared to the control, chironomids (*Chironomidae*) exposed to glyphosate were represented by specimens smaller in length while *A. aquaticus* only showed large adults. The ranges of glyphosate concentration in the tissues of sampled macroinvertebrates and *Phragmites australis* organs were 7.3-10.2 μg/kg and 16.2-24.7 μg/kg, respectively.

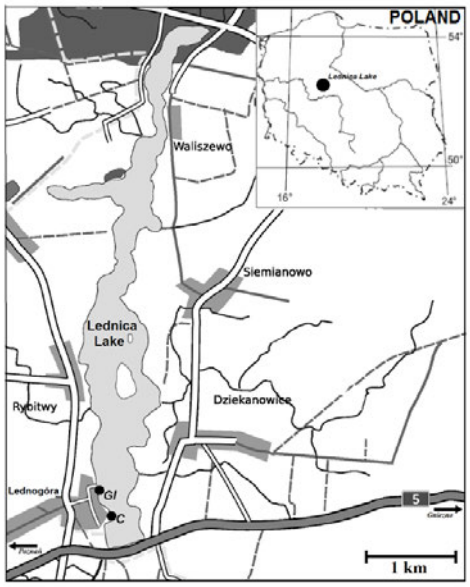
Materials and methods

Study site
Lake Lednica is situated within a protected area of Lednica Landscape Park in the southern part of the Gniezno Lakeland about 35 km east of the City of Poznań in west-central Poland. Lake Lednica covers an area of 339.1 ha and fills the southern part of a tunnel valley which extends between Janowiec and Lednogóra. It is an elongated water body with a total length of 7.3 km, maximum width of 825 m, and maximum depth of 15.1 m. Several small streams flow into the lake. The catchment area covers over 384 km^2 dominated by cultivated lands.

Sampling
The sampling site was situated at the bathing area of Lake Lednica – near the village of Lednogóra. Samples of emerged plants – *Phragmites australis* (Cav.) *Trin. ex Steud*. – were collected manually from a 0.5 m × 0.5 m plot. Leaves, stems and roots were stored separately in a freezer at - 20ºC. Water was collected from a depth of 0.5 m in a plastic bottle. In the laboratory water was filtered through a GF/C filter. Benthic macroinvertebrates were collected together with sediments using a littoral core sampler. For each station 17 subsamples were taken. At both stations subsamples were collected from similar

habitats – within submerged macrophytes. The collected material was sieved through a 0.25 mm mesh. After taxonomical identification, individuals were frozen at -20 ºC.
As control samples from a site located 200 m from the event site were collected (Figure 1). No visible signs of plants withering or other potential toxic effects of herbicide were observed.

Figure 1. The study sites and location of Lake Lednica. Gl – glyphosate treated site, C – control site



Chemical analysis
Glyphosate concentration in water and biota was determined using a gas chromatography – mass spectrometry (GC/MS) system. Capillary column Agilent Hp-5ms (0.32 mm ID × 30 m length × 0.25 µm thickness film) was applied. The total run time was 18 min. The injection volume was 2 mm^3 with split 1:80. Helium maintained at a constant flow rate of 0.5 cm^3/min was used as the carrier gas. Standard glyphosate (IPO, Poland) was dissolved in distilled water (250 cm3). Calibration solutions (0.1, 0.5, 2.5, 5.0, 10.0 and 20 µg/cm^3) were made by dilution.

Homogenized macroinvertebrates and plant samples (2-3 g) were shaken separately for 30 minutes with 25 cm^3 of 2M NH_4OH and centrifuged at 5000 rpm for another 30 min. The supernatant fractions were evaporated to dryness under vacuum. Water samples (25-30 cm^3) were evaporated. The residues were dissolved in 6 cm^3 of water-methanol-hydrochloric acid (40:10:1) with the addition of 40 cm3 of 85% phosphoric acid. Samples were then incubated at 20 °C for 2 h and centrifuged at 5000 rpm for another 30 min. Supernatants were evaporated in flasks equipped with a reflux condenser protected by a drying tube. After chilling the flasks (4 °C) and adding the derivatization reagents – 2 cm^3 of trifluoroacetic anhydride (TFAA) and 1 cm^3 of 2,2-trifluoroethanol (TFE) – the mixture was held for 1 h in an oil bath at 100 °C. The excess of derivatization reagents was removed by evaporation. The flask was rinsed with distilled water and methylene chloride and solvents were transferred to a separator funnel. After shaking for 2 min, the organic layer was collected. The extraction process was performed in triplicate.

Results

Chemical analysis
Glyphosate was detected in water collected from the bathing area of Lake Lednica. Mean determined concentration was 90 ± 10.0 µg/dm^3. No glyphosate was found in water at the control station.
Concentrations of glyphosate in *Phragmites australis* collected from the treated site were found with the highest values observed for leaves at 24.7 µg/kg (Table 1). Glyphosate content in *P. australis* tissues was at least 3 times lower than that found in parallel water samples.
Low glyphosate content in macro-invertebrates with the highest level were determined for chironomids

at 10.2 µg/kg. At the same time no glyphosate was found in any sample collected from the control site. The highest level of glyphosate was found in tissues of *chironomids* while the lowest in gastropods.

Table 1. Mean glyphosate content (µg/kg1) found in macroinvertebrates and *Phragmites australis* collected from Lake Lednica in April, 2011 (n=3).

	Bathing area	Control
Macroinvertebrates		
Chironomids	10.2	n.d.
Gastropoda	7.3	n.d.
Other	8.1	n.d.
Phragmites australis		
Leaves	24.7	n.d.
Stems	20.9	n.d.
Roots	16.2	n.d.

n.d. – not detected; Other: *Ephemeroptera* + *Megaloptera* + *Trichoptera*

Biological results

No significant differences in taxa number between the bathing area (12 taxa) and the control site (13 taxa) were determined although there was a difference in abundance of specimens and taxonomic composition.

The density of the benthic macroinvertebrates found at the bathing area was over 40 % lower than at the control site (Table 2). A significantly lower abundance of midges from the family *Chironomidae* (41 %), *Oligochaeta* (43 %), *Viviparіae* snails (75 %), leeches (75 %), freshwater crustaceans: *Asellus aquaticus* (77 %), *Gammarus pulex* (38%) and zebra mussel *Dreissena polymorpha* (42 %) were found.

The area treated with glyphosate was characterized with a higher number of Gastropoda.

Table 2. Differences in the density (individuals per square metre) of benthic macroinvertebrate taxa found at the bathing area and control site in Lake Lednica.

	Bathing area	Control
Oligochaeta *	300	525
Hirudinea *	100	400
Tricladida		
- Dendrocoelum sp.	–	75
Crustacea		
Amphipoda *		
- Gammarus pulex	325	525
Isopoda		
- Asellus aquaticus *	450	1975
Insecta		
- Trichoptera *	450	600
- Megaloptera *	875	975
- Ephemeroptera *	275	475
Diptera		
-Chironomidae *	2375	4025
- Ceratopogonidae	50	50
Bivalvia		
- Dreissena polymorpha *	1325	2300
Gastropoda		
-Viviparіae *	50	200
-Lymnaeidae	25	–
Total	6575	12125

* – statistical difference between studied sites (Mann-Whitney U, $p<0.05$)

Conclusion

Glyphosate was detected in the water samples collected from the bathing area at a mean concentration of 0.09 mg/dm^3. Aboveground parts of emerged macrophytes (*Phragmites australis* and *Typha latifolia*) covering the investigated area were completely withered. Studies of benthic macroinvertebrates revealed no significant differences in taxa number between event (13 taxa) and control (14 taxa) sites although differences in abundance of particular taxa were observed. Significantly lower numbers of *Chironomidae* (by 41 %), Oligochaeta (by 43 %), Viviparіae (by 75 %), Hirudinae (by 75%), *Asellus aquaticus* (by 77 %), *Gammarus pulex* (by 38 %) and *Dreissena polymorpha* (by 42 %) were found at the glyphosate-treated site. Furthermore, compared to the control, chironomids (*Chironomidae*) exposed to glyphosate were represented by specimens smaller in length while *A. aquaticus* only showed large adults. The ranges of glyphosate concentration in the tissues of sampled macroinvertebrates and *Phragmites australis* organs were 7.3-10.2 µg/kg and 16.2-24.7 µg/kg, respectively.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: This paper describes the impact of an episodic pollution event on Lake Lednica in Poland, describing monitoring results for glyphosate detects in lake water and the apparent impact on various aquatic taxa groups, in comparison to a control station site established at another location. These data cannot be related to a specific use or application rate and therefore are not relatable to the EU ecotoxicological regulatory risk assessment for the glyphosate EU renewal.

Assessment and conclusion by RMS:

This paper describes the impact of an episodic pollution event on Lake Lednica in Poland, describing monitoring results for glyphosate detects in lake water and the apparent impact on various aquatic taxa groups, in comparison to a control station site established at another location.
Glyphosate concentrations were measured.
Studies of benthic macroinvertebrates revealed no significant differences in taxa number between event (13 taxa) and control (14 taxa) sites although differences in abundance of particular taxa were observed.
RMS notes that differences between sites were likely even before the pollution event. No data was available to confirm that abundances in both sites were similar (before the event).
Relevant but not reliable for regulatory risk assessment.

Data point:	CA 9
Report author	Canosa I. S. *et al.*
Report year	2018
Report title	Ovarian growth impairment after chronic exposure to Roundup Ultramax® in the estuarine crab *Neohelice granulata.*
Document No	Environmental science and pollution research international (2018), Vol. 25, No. 2, pp. 1568-1575
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Adult females of the estuarine crab *Neohelice granulata* were exposed to the glyphosate formulation Roundup Ultramax® during the entire 3-month pre-reproductive period. At the end of the assay, a significant higher increment of glycemia was noted at both glyphosate concentrations assayed (0.01 and 0.2 mg/L, acid equivalent). Although no differences were observed in the gonadosomatic index, a significantly higher proportion of reabsorbed vitellogenic oocyte was observed at the highest glyphosate concentration, together with a significant decrease of vitellogenin content in the ovary. In addition, some *in vitro* assays were carried out by co-incubating small pieces of ovary with or without the addition of Roundup Ultramax®; at both concentrations tested (same as those used *in vivo*), a decrease in the ovarian vitellogenin content was observed, whereas the ovarian protein synthesis was significantly inhibited by glyphosate at 0.2 mg/L in the Roundup Ultramax® formulation used.

Materials and methods

A) In-vivo method

Test substances:	Roundup UltraMax (67.9% a.e. purity).
Exposure concentrations:	Control, 0.01 and 0.2 mg a.e./L
Test species*:*	Females estuarine crabs *Neohelice granulata.*
Size:	body weight = 10.69 ± 0.09g
Age:	Adult
Source:	Wild collected from Southern edge of Samborombón Bay
Test design:	Semi-static – volume completely replaced twice a week.
Test duration:	3 month exposure period
Replication:	15 females per treatment and control, contained in individual glass containers.
Animal per replicate:	Single crabs per container
Test volume (L):	400 mL glass container.
Feeding:	Lab prepared food pellets twice a week, fed at 5% of body mass,.
Chemical analysis:	In order to validate nominal glyphosate concentrations, water samples (15 mL) were taken at 0 and 72 h, i.e., the period for water replacement in all test containers. After derivatization at pH=9 with 9-fluorenyl-methylchloroformate (FMOC-CL), glyphosate concentrations were measured by high-pressure liquid chromatography, coupled to an Agilent mass spectrophotometry detector, model VL. An X-SELECT C18 chromatographic column was used. A mixture of MeOH/NH_4 (5 mM) 9 mM was chosen as mobile phase, with a flow rate of 0.5 mL/min.
Environmental conditions:	Water hardness 80 mg/L as. equivalents of $CaCO_3$; final salinity, 12 g/L, pH=8.0 ± 0.5. Constant aeration. A temperature of 23 ± 1 °C and a photoperiod of 14:10 (L/D) were maintained throughout.

B) In-Vitro method

Stock female crabs (maintained under the same conditions used for controls of the *in vivo* assay) were used for the *in vitro* experiments. Ovarian explants (approximately 1 × 0.5 cm each) were incubated for 24 h in CO_2 chambers at 27 °C and in constant darkness. Each female provided a similar piece of ovary for one vial of every group of the same experiment (blocked design). Medium 199 was prepared using powdered medium with L-glutamine and Earle's salts (Sigma Chemical Co.), dissolved in crustacean saline (Cooke et al., 1977), and modified to compensate for the salts already present in the culture medium. In addition, as in previous studies (Sarojini et al. 1997; Rodríguez et al. 2000), 6 mg of penicillin-G per 100 mL of medium was added to prevent bacterial growth, and the pH was adjusted to

7.4 with 0.5 N NaOH. Vitellogenin content of ovarian explants was measured in two independent experiments run with glyphosate added to the culture medium at a concentration of 0.01 or 0.2 mg/L, together with a paired control with no glyphosate added. At the end of the 24-h incubation period, the total content of vitellogenic proteins (vitellogenin and vitellins) was determined using the same methodology described for the *in vivo* assay. In a third *in vitro* experiment, made at the same glyphosate concentrations and control above mentioned, tritiated leucine was added to the culture medium at the start of the incubation in order to estimate the synthesis of ovarian protein by its incorporation to the acid-precipitable protein fraction, following the methodology previously used for *N. granulata* and other crustacean species. Briefly, an aliquot of 30 µL from the 3H-leucine stock solution (1:10 dilution from 1 mCi/mL; NEN Life Science, Inc.) was added to each vial, to reach a total activity of 3 µCi. At the end of the incubation period, the ovarian explants were weighed and homogenized in 2 mL cold 10% trichloroacetic acid (TCA) followed by centrifugation at 5000×g for 10 min at 4 °C, then washed twice with cold TCA, resuspended, and decanted into a Millipore suction filtration funnel using 0.22-µm nitrocellulose filter disks. After filtration, the disks were air-dried for 1 h and submerged in scintillation fluor solution (Optiphase Hi Safe 2) overnight at 4 °C. Radioactivity was measured in a Beckman scintillation counter. Uptake of labeled leucine by the ovary was expressed on an ovarian wet-weight basis (CPM/mg ovary).

Statistical analysis: Proportion of survival, molted, or ovigerous females were compared between experimental groups by the Fisher exact test (Sokal and Rohlf 1981). For all the continuous variables analyzed, a one-way ANOVA followed by LSD multiple comparisons (Sokal and Rohlf 1981) was used for testing differences between experimental groups and control. When no homogeneity of variance was confirmed, the variance heterogeneity was incorporated to the statistical model by using the R studio 3.3.1 program (Zuur et al., 2009).

Results

Measured concentrations of glyphosate were close to the nominal. Low mortality was observed (up to 6.7%) in the *in vivo* experiment, while the percentage of females that became ovigerous was as high as 20%. No significant differences were detected.

Table 1. Nominal versus measured concentrations of glyphosate (as acid equivalent) in the Roundup Ultramax® formulation.

Glyphosate concentration (mg/L)	Measured concentration (mg/L)		
	0 h	72 h	Overall mean
0 (control)	0.0000 0.0000	0.0000 0.0000	0.0000
0.01	0.0136 0.0111	0.0090 0.0083	0.0105
0.2	0.1886 0.2075	0.1564 0.1523	0.1762

Table 2. Survival, molting, and ovigerous percentages at the end of both experiments.

Glyphosate concentration (mg/L)	Ni	% mortality	% molting	% ovigerous	Nf
0 (control)	15	0.00	0	20	12
0.01	15	6.67	0	13.33	12
0.2	15	13.33	6.67	20	9

No significant differences ($p < 0.05$) with respect to control were observed, in any case

Ni initial number, *Nf* final number, considering only the non-molted, non-ovigerous surviving females

Concerning energy reserves, no differences ($p > 0.05$) were observed in the muscle in either protein (overall mean = 38.99 ± 2.56 mg/g) or lipid content (overall mean = 1.17 ± 0.12 mg/g); although not statistically significant ($p > 0.05$), a clear decrease in muscle glycogen content was observed at the highest concentration of glyphosate, compared to control, concomitantly with a significantly higher ($p < 0.05$) increase of glycemia. Vitellogenin levels are shown in for the *in vivo* assay. A significantly ($p < 0.05$) lower vitellogenin content was noted at the highest glyphosate concentration. No differences among treatments ($p > 0.05$) were detected in the level of circulating vitellogenin, in any case. No significant ($p < 0.05$) differences among treatment were noted in the gonadosomatic index (overall mean = 2.34 ± 0.47). At 0.2 mg/L, a significantly ($p < 0.05$) higher proportion of reabsorbed vitellogenic oocyte was observed. Although not statistically significant ($p > 0.05$), a lower area was observed in the vitellogenic normal oocytes from females exposed to 0.2 mg/L of glyphosate.

Conclusion

The authors concluded that Roundup Ultramax®, at concentrations within the environmental range, was able to reduce the vitellogenin content of the ovary during the pre-reproductive maturation period. Such reduction showed to be related to oocyte reabsorption, observed *in vivo*, as well as to the inhibition of protein synthesis in the ovary, observed in vitro. Additionally, the studied herbicide caused a marked increment in glycemia at the end of the in vivo exposure

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Roundup Ultramax® is not the representative formulation for the glyphosate EU renewal. Therefore the article is not relevant for the renewal. In addition, cellular and molecular level endpoints are discussed that cannot be used in the EU level ecotoxicological regulatory risk assessment.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

This study originates from the same lab that produced the following study:
Avigliano L. et al., 2014; Effects of glyphosate on egg incubation, larvae hatching, and ovarian rematuration in the estuarine crab Neohelice granulate.

Avigliano L. et al., 2014 stated that based on a comparison of number of hatched larvae per female between Roundup Ultramax (clear embryonic mortality) and glyphosate (no significant increase of mortality) at equivalent concentration, Roundup compounds other than glyphosate may be responsible for the embryonic mortality.
RMS then doubts the relevance of the data in this study (Canosa I. S. et al., 2018) as only Roundup Ultramax was used and no discrimination between glyphosate and other compounds is feasible.

The study is considered not relevant.

Data point:	CA 9
Report author	Magbanua F. S. *et al.*
Report year	2013
Report title	Understanding the combined influence of fine sediment and glyphosate herbicide on stream periphyton communities.
Document No	Water research (2013), Vol. 47, No. 14, pp. 5110-20
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: **as provided in the AIR5 dossier (KCA 9) as proposed by applicant:** **See RMS analysis in RMS comment box**	Not relevant by full text

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: This study investigated the combination of sediment and glyphosate effects on an mesocosm community. This paper presents no results that can be used in an EU level risk assessment for Annex I renewal purposes.
The test substance was identified as Glyphosate commercial formulation with 360 mg/L active ingredient plus containing 10-20% polyethoxylated tallowamine (POEA). POEA surfactant is not permitted for use in formulated herbicidal products in the EU. As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation for the glyphosate EU renewal, and are therefore not relevant for the renewal. The surfactant system used in the formulation tested in this paper is POEA based, whereas the surfactant used in the representative formulation for the glyphosate EU renewal (MON 52276) is quaternary-ammonium based.

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

In this study, it is clearly stated by the study authors that the tested formulation included the surfactant polyethoxylated tallow amine (POEA). Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Therefore it is not possible to discriminate between glyphosate and POEA.
The study is considered not relevant by RMS.

7. Endocrine disruption

Data point:	CA 8.2.3
Report author	Xia S. et al.
Report year	2013
Report title	Induction of vitellogenin gene expression in medaka exposed to glyphosate and potential molecular mechanism
Document Source	Zhongguo Huanjing Kexue (2013), Vol. 33, No. 9, pp. 1656-1663
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

To demonstrate the estrogenic activities of glyphosate and clarify the underlying molecular mechanism, 1~3days old Japanese medaka were exposed to 0.2, 2, 20, 200, 2000 μg/L of glyphosate for 5weeks. Transcription levels of vitellogenin (VTG I) and enzyme genes involved in 17β-E2biosynthesis and metabolism were measured by Q-RT-PCR. While glyphosate markedly up-regulated VTG transcription levels in both female and male fish, the upward trend was inhibited at the high glyphosate concentrations (at 200 μg/L and 2000 μg/L for female, at 2000 μg/L for male). The gender specific molecular mechanism was observed. In female fish, VTG induction would be due to the increase of transcription levels of FSH gene in brain and CYP19A gene in the gonad, and therefore enhancing the biosynthesis of 17β-E2. However, in male fish, the inhibited CYP1A, CYP1B and CYP3A transcription levels, which would inhibit 17β-E2 metabolism, would be the reasonable explanation.

Materials and Methods

Materials and Reagents
40 wt.% Glyphosate isopropylamine salt solution was purchased from Sigma; trizol was purchased from Invitrogen; M-MMLV was purchased from Beijing ComWin Biotech Co., Ltd.; SYBR Green PCR Master mix was purchased from Invitrogen.

Feeding of Experimental Fish
Orange-Red strains are bred and raised in our laboratory, and the water for breeding was the aerated tap water filtered by activated carbon. Water temperature was controlled at (25 ± 1)℃. The water hardness (CaCO3) was (81.1 ± 1.2) mg/L. The pH value was 7.9 ± 0.1. The dissolved oxygen was (7.8 ± 0.3) mg/L. The photoperiod was 16h:8h (Day:Night).The larvae just hatched by Artemia nauplii were used as the bait, and they were fed once every morning and afternoon. The excess feed and excreta were removed in time.

Exposure Experiment
The fish fries which were just hatched 1 ≤ 3 days (1~3 dph) were exposed to the experiment. A total of 5 concentration gradients were set, and the concentrations of glyphosate isopropylamine salt in each gradient were 0.2, 2, 20, 200, 2000 μg/L, respectively. Tap water, which was filtered by activated carbon and aerated, was used as blank control. Change water in full quantity every day. A series of standard samples with concentrations of 0.004, 0.4, 4.4, 40 μg/L were prepared by dilution of 40 wt. % glyphosate isopropylamine solution with ultra-pure water. 200 μL of the above standard samples were taken into the corresponding 4 L exposure pool, and the final concentrations of the exposure pool were 0.2, 2, 200, 2000 μg/L, respectively.
The exposure lasted for 5 weeks. On the last day, six male and female fish in each group were randomly anesthetized with ice, and then the liver, gonad and brain of each fish were taken, marked and put into liquid nitrogen to extract total RNA.

Total RNA Extraction and cDNA First Chain Synthesis
The total RNA of liver, brain and gonad was extracted respectively by Trizol reagent, and then the total RNA was treated and purified by DNase I. The UV absorption of A 260 ≤ 280 by total RNA is more than 1.8. The cDNA first chain was synthesized by HiFi-MMLV reverse transcriptase and OligodT15 primers.

Fluorescence Quantitative PCR Detection
According to the method established in our laboratory, the fluorescence quantitative PCR was detected by ABI7500 fluorescence quantitative PCR. RPL-7 was used as the internal reference gene to correct experimental errors. Primer Premier 5.0 (Premier Biosoft) designed the primer sequence of the tested gene, and crossed the intron as much as possible in order to reduce DNA pollution. The specific sequence of the primers is shown in Table 1, synthesized by Beijing Sunbiotech co., Ltd Gene Primer Sequence Genebank NO.

Table 1: Primer pairs of SYBR® Green real-time PCR

FSHβ	5'-GGGATGTCCAGTGGGTTTCA-3' 5'-GCCGCAGTATGTGGTTCTTGT-3'	EF535801
CYP19A	5'-GCGTAGAGCCCTTTTCGATGA-3' 5'-TGCGGCCCGTATTCAAGAT-3'	D82968
17βHSD Ⅰ	5'-CTTGGCTGGAATGAAAGCACA -3' 5'-TGAAAGGAAGCCCATGGAGTC -3'	EF530597
VTG Ⅰ	5'-CTCCAGCTTTGAGGCCATTTAC-3' 5'-ACAGCACGGACAGTGACAACA-3'	AB064320
CYP1A	5'-ATCGGCCTGAATCGAAATCC-3' 5'-TGTGTCCCTTGTTGTGCAGTGT-3'	AY297923
CYP1B	5'-GCTGTTTCTCTTCGTGGCATTA-3' 5'-CGATGTCATAGGCGTGAGGTTT-3'	JF894387
CYP3A	5'-GAGATAGACGCCACCTTCC-3' 5'-ACCTCCACAGTTGCCTTG-3'	AF105018
RPL-7	5'- CGCCAGATCTTCAACGGTGTAT -3' 5'- AGGCTCAGCAATCCTCAGCAT -3'	DQ118296

Data Processing

Quantitative PCR data were used to calculate the relative expression of genes by 2-ΔΔCt method. The independent sample t test of SPSS software (Ver 11.5; Chicago, IL, USA) was used to test the significance of differences in gene expression between different exposure groups and control groups ($P < 0.05$). The gene expression in different concentration exposure groups of each gene was expressed by the multiple of gene expression in the control group.

Results

Changes of Vitellogenin Gene Expression in Liver

In female juvenile fish, glyphosate at low concentration (0.2 μg/L), could significantly induce the expression of vitellogenin gene in female fish, but the expression of VTG I decreased gradually with the increase of exposure concentration, showing an apparent dose-effect relationship. With the increase of exposure concentration, the gene expression of VTG in each concentration group was 101.36, 41.76, 11.92, 5.26 and 5.34 times higher than that in the control group, respectively (Fig. 1a). In the high concentration group (200, 2000 μg/L), although there was also increase of VTG I gene expression as compared with the control group, but the increase was not obvious. In male fish, the VTG I gene expression induced by glyphosate increased first and then decreased: VTG I gene expression at the concentration of 0.2 μg/L, 2 μg/L, and 20 μ/ L was 1.64, 2.87, and 41.35 times, respectively, higher than that of the control group.

When the concentration of glyphosate increased to 200, 2000 μg/L, the expression of, VTG I was 13.92 and 1.31 times higher than that of the control group, respectively (Fig. 1b). Except the lowest exposure group (0.2 μg/L) and the highest exposure group (2000 μg/L), the VTG I in all other groups was significantly induced.

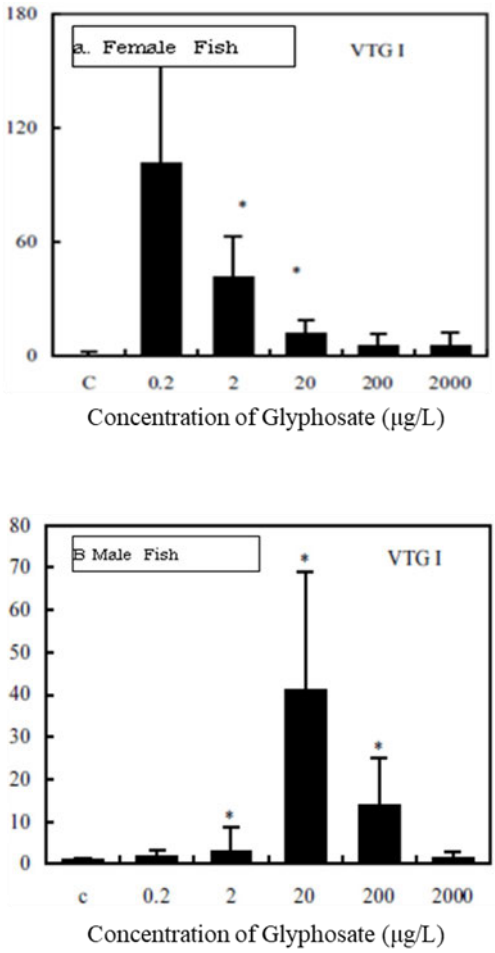


Figure 1: Vitellogenin gene expression in liver of medaka exposed to glyphosate (*P<0.05; n = 4~6)

Changes in Estrogen Synthesis and Metabolic Enzyme Gene Expression

- *Changes in Gene Expression in Pituitary Gonadal Axis*

Fifteen genes in the pituitary gland axis were detected. Among them, three Hypothalamic gonadotropins release hormone (cGnRH, mdGnRH, sGnRH) genes and three pituitary gonadotropin subunits (GtH α, FSH β, LH β) genes,regulating the synthesis and secretion of pituitary gonadotropins and gonadal sex hormones, respectively. Besides, nine genes related to hormone synthesis (StaR, HMGR, CYP11A, CYP11B, CYP17, CYP19A, 3 β-HSD, 17 β-HSDI, 17 β-HSD III) were also involved. In the early stage of steroid hormone synthesis, cholesterol synthesis is mainly regulated by hydroxymethyl glutaryl coenzyme A reductase (HMGR). Steroid acute regulatory protein (StAR) is responsible for transporting cholesterol into mitochondria, initiating the synthesis steroid hormones. CYP11A catalyzes cholesterol to form pregnenolone, which is the starting point of steroid hormone biosynthesis, and then catalyzed by 3 β-HSD enzyme to synthesize progesterone. CYP17 enzyme has 17 α-hydroxyenzyme and 17, 20-lyase activities, which can catalyze the production of 17 α-hydroxyprogesterone and androstenedione, rexpetively, synthesizing the two precursors of downstream estrogen. CYP19A and CYP11B regulate the synthesis of estrogen estrodiol (E2) and androgen 11- ketotestosterone (11-KT). 17 β-HSD I can convert estrone into estrodiol, which is more bioactive. 17 β-HSD III is the metabolic enzyme of androgen, which reduces androdione to testosterone. These 15 genes mainly cover the whole process of sex hormone synthesis regulated by pituitary gland axis. This study used quantitative PCR detection to screen out the genes which are significantly affected by glyphosate in order to further explain the molecular mechanism of the estrogen effect of glyphosate.
Results show that in female fish, from the low concentration group to the high concentration group, the

relative expression of FSH β mRNA in brain was 3.71, 1.19, 0.87, 0.72 and 0.90 times higher than that in control group, respectively. As shown in Fig. 2a, the expression of FSH β in female fish was significantly increased ((P<0.05) under 0.2 μg/L glyphosate exposure, but there was no significant change in other concentration groups. The relative expression of CYP19A and 17 β-HSD I genes in gonads was 2.16, 1.33, 1.18, 1.42, 0.77 times (Fig. 2c) and 1.49, 1.95, 1.19, 1.25, 0.87 times as much as that in the control group, respectively (Fig. 2e). 17β-HSDI The CYP19A gene expression was significantly induced by 0.2 μg/L glyphosate concentration, but the expression of other concentration groups did not change significantly compared with the control group but decreased gradually with the increase of concentration. 17 β-HSDI gene expression was significantly induced at 2 μg/L glyphosate concentration, but this gene expression had no significant change in the other concentration groups. From the low concentration group to the high concentration group, the relative expression of FSHβ mRNA was 0.91, 1.19, 1.85, 1.12 and 1.23 times of the control group (Fig. 2b), but it was only induced when at 20 μg/L (P<0.05); The relative expression of 17 β-HSDI gene in male fish was 2.31, 2.90, 4.33, 7.98, 3.08 times of the control group (Fig. 2f). At the concentrations of 0.2μg/L and 2000 μg/L, the expression of 17 β-HSDI was significantly increased (P<0.05), and the expression of 17 β-HSDI gene in other groups showed a rising trend, which was more than 2 times different from the control group. However, the difference was not significant.

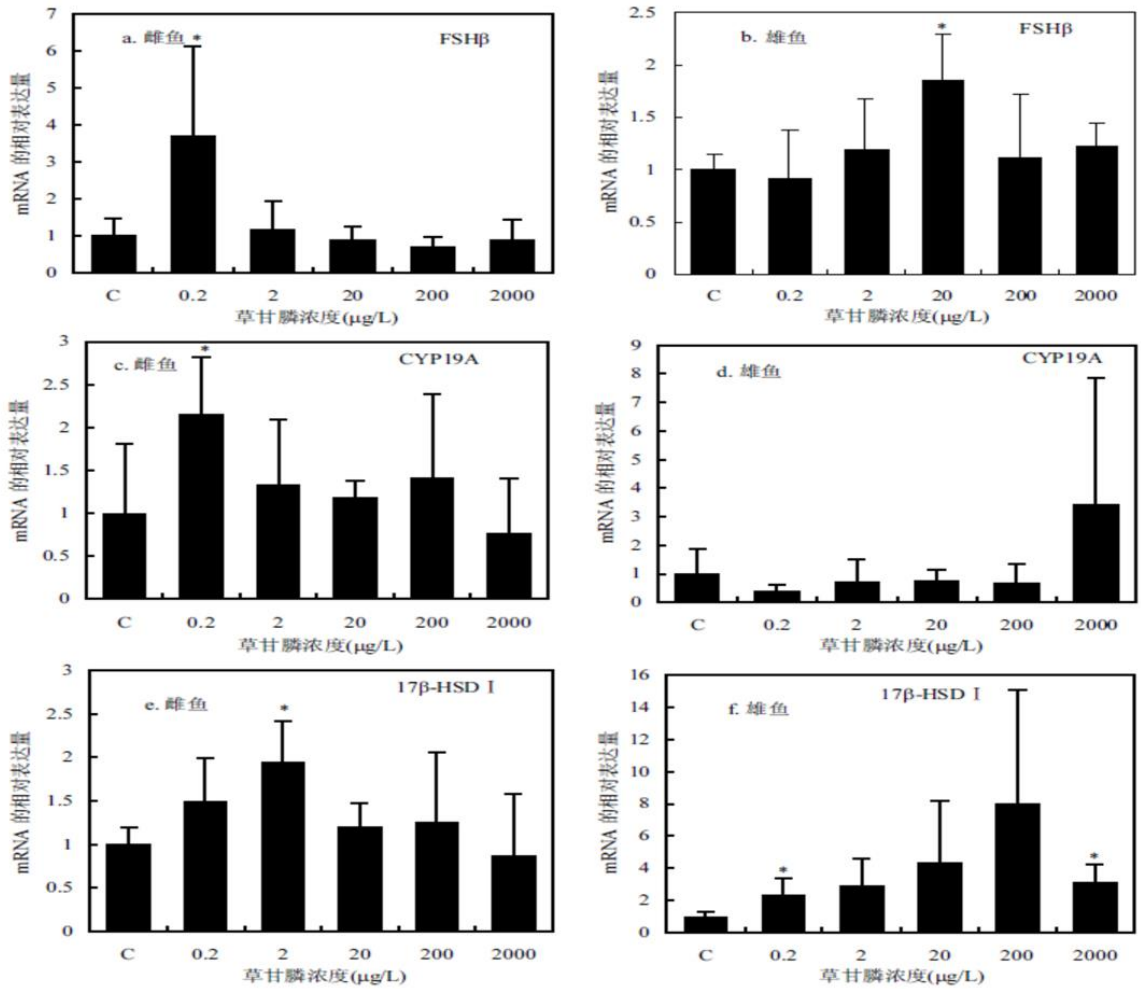


Figure 2: Hypophysical – gonadal axis key genes expression of medaka exposed to glyphosate (*P<0.05; n = 4~6) (in the 6 charts, a., c, e, are female fish; b., d, f, are male fish)
(Y axis – mRNA relative expression; X axis –glyphosate concentration (μg/L))

Changes of Gene Expression of Estrogen Metabolizing Enzyme

Glyphosate exposure in each concentration group could significantly inhibit the expression of estrogen

metabolizing enzyme gene in female fish ($P < 0.05$). The expression of CYP1A, CYP1B and CYP3A mRNA in the order of increasing concentration was 0.42, 0.19, 0.27, 0.26 and 0.66 (CYP1A); 0.06, 0.35, 0.15, 0.26, 0.11 (CYP1B); 0.31, 0.20, 0.23, 0.18, 0.21 (CYP3A) times as much as that in the control group, respectively (Figures 3a, 3c and 3e). With the increase of exposure concentration, the expression of CYP1A decreased first and then increased, while the expression of CYP1B and CYP3A was relatively stable.

As shown in Figs. 3b, 3d and 3f, the expression of CYP1A mRNA in male fish was 0.38, 0.12, 0.27, 0.17, 1.16 times respectively the expression in the control group from low to high concentration, and the expression of CYP1B was 0.44, 0.15, 0.36, 0.42, 2.94 times, respectively; and the expression of CYP3A was 0.55, 0.32, 0.38, 0.45 and 0.70 times, respectively. In addition to the fact that there was no significant difference between the expression of CYP1A and CYP1B in the highest concentration group and that in the control group, the expression of the liver estrogen-metabolizing enzyme in the male fish was significantly inhibited in other concentration groups ($P < 0.05$). Also, with the increase of the exposure concentration, the increased- first- and- decreased- afterwards U-shape expression pattern was presented.

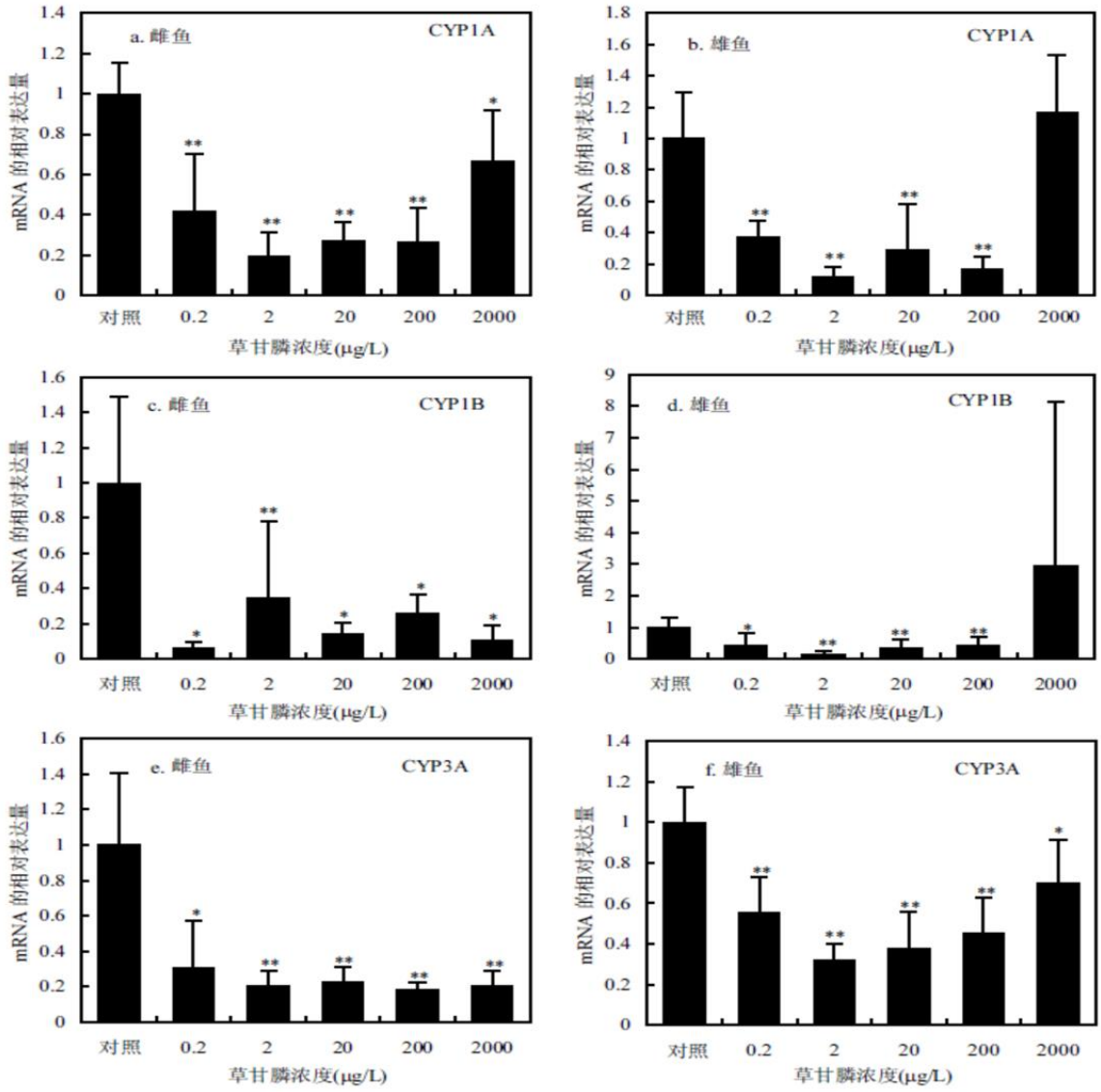


Figure 3: Expression of estrogen-related metabolic genes in liver of medaka exposed to glyphosate
(in the 6 charts, a., c, e, are female fish; b., d, f, are male fish)
(Y axis – mRNA relative expression; X axis –glyphosate concentration (μg/L))

Conclusion

In this study, Glyphosate exposure could significantly induce the expression of VTGI gene in female and male Medaka, and showed a dose-effect relationship (Fig. 1), indicating that glyphosate had a certain estrogen effect. The induction ability of VTGI genes in male fish was obviously lower than that in female fish, which means, female fish were more sensitive to estrogens than male fish. In order to further analyze the potential mechanism of VTGI expression induced by glyphosate, this study has measured the expression changes of estrogen synthesis and metabolic-related genes. Aromatase (CYP19) and 17 β-hydroxysteroid dehydrogenase (17 β-HSDI) are two important enzymes that promote gonadal estrogen synthesis. They can convert testosterone (Testosterone, T) into 17 β-E2 and estrone (Estrone, E1) into 17 β-E2. In glyphosate female fish 2 μg/L exposure group, 17 β-HSDI was significantly expressed in gonad ($P < 0.05$), which may contribute to the increase of VTG. In vertebrates, CYP1A, CYP1B and CYP3A in liver cytochrome 450 enzyme (CYP450s) are the main enzymes that metabolize 17 β-E2 to 2-hydroxyestrodiol (2-OHE2). Studies have shown that CYP1A plays a more important role in 17 β-E2 metabolism in fish. Results have shown that the expression of FSH β and gonadal CYP19A in the brain of female fish was significantly expressed at the lowest concentration ($P < 0.05$), and then decreased with the increase of exposure concentration, showing an expression pattern of VTG which is similar to that of female fish. This may be due to the fact that FSH in the brain can increase the expression and activity of gonadal aromatase (CYP19A), which increases the synthesis of gonadal estrogen, resulting in the increase of VTG synthesis. On the other hand, estrogen metabolizing enzymes in the liver of female fish were significantly inhibited in all exposure concentration groups ($P < 0.05$), and this inhibitory action was similar to the report that chlorpyrifos inhibited the expression of CYP1A. Similar to female fish, the expression of estrogen metabolizing enzyme in the liver of male fish was also significantly inhibited. In addition, CYP1A, which played a greater role in E2 metabolism, showed U-type expression pattern, which was consistent with the dose- effect relationship of inverted U-type expression of the VTG genes in male fish. According to Anderson et al., the expression of CYP1A in rainbow trout hepatocytes was negatively correlated with the synthesis of VTG. Therefore, glyphosate inhibited estrogen metabolism in male fish and induced VTG gene expression in the liver of male fish. Besides the metabolic factors, while the expression pattern of FSH β in the brain of male fish was the same as that of VTG, gonadal CYP19A genes did not show significant changes in all concentration groups. The expression of 17 β-HSDI in male fish was significantly higher than that in the lowest and highest concentration groups ($P < 0.05$), but there was no significant change in the expression between the two groups. In the experimental group where VTG expression significantly increased, the expression of 17 β-HSDI in male fish was of more than twice as much, but there was no significance due to the great differences within the group. It is indicated that 17 β-HSDI may not play a key role in the rise of VTG.

Glyphosate can induce VTG expression in Medaka in a certain concentration range, showing estrogen effect. Also, there are certain differences in the induction mechanism of estrogen effect between male and female fish. In female fish, glyphosate can up-regulate the expression of FSH to induce the expression of CYP19A, thus increasing the ability of estrogen synthesis. However, in male fish, the change of VTG expression was induced by inhibiting estrogen-metabolizing enzymes (CYP1A and CYP3A) in the liver resulting in the increase of the 17 β-E2 concentration in vivo.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study was not conducted according to GLP and a relevant guideline was not followed. The current EU stepwise endocrine approach is detailed, and the approach conducted within this study does conform to the suggested guidance. Significant limitations in the study include a lack of a standard testing approach or specific validation criteria. The test concentrations were not analytically verified and the critical dose regime provided to the Medaka is lacking. Similarly the source of the fish tested is unknown. No clear dose response relationship or derived endpoint from the study could be determined.

Further points of clarification:
The test substance used in the study is not sufficiently identified, but based on the stated '40 wt.% glyphosate isopropylamine salt solution content', this is not the representative formulation for the glyphosate EU renewal.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:
Original report in Chinese, results are based on a document translated in english.

To demonstrate the estrogenic activities of glyphosate and clarify the underlying molecular mechanism, 1-3 days old Japanese medaka were exposed to 0.2, 2, 20, 200, 2000 µg/L of glyphosate for 5 weeks. Transcription levels of vitellogenin (VTG I) and enzyme genes involved in 17β-E2 biosynthesis and metabolism were measured by Q-RT-PCR.

This study states that glyphosate (in formulation) can induce VTG expression in Medaka in a certain concentration range, showing estrogen effect. This was however inhibited at the high glyphosate concentrations (at 200 µg/L and 2000 µg/L for female, at 2000 µg/L for male). Also, there are certain differences in the induction mechanism of estrogen effect between male and female fish. In female fish, glyphosate can up-regulate the expression of FSH to induce the expression of CYP19A (aromatase), thus increasing the ability of estrogen synthesis. However, in male fish, the change of VTG expression was induced by inhibiting estrogen-metabolizing enzymes (CYP1A and CYP3A) in the liver resulting in the increase of the 17 β-E2 concentration in vivo.

A formulation was used and co-formulants are not stated. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product.
The study does not follow a standardized approach.
No analytical verification available. No information is provided about the toxicity of the tested doses (no data was available on the mortality, bodyweight, growth, etc…). Then it is not possible to determine whether the effects on protein expression are a result of general systemic toxicity.
No biological data presented, only graphs.

The study is relevant but of low reliability. It is proposed by RMS to not include it in the assessment of potential for endocrine disruption.

Data point:	CA 8.2.1
Report author	Druart C. *et al*.
Report year	2017

Report title	A full life-cycle bioassay with *Cantareus aspersus* shows reproductive effects of a glyphosate-based herbicide suggesting potential endocrine disruption.
Document Source	Environmental pollution (2017), Vol. 226, pp. 240-249
Guidelines followed in study	EPPO (2003) Chapter 8: Soil organisms and functions OECD Series on Testing and Assessment No. 95 OECD Series on Testing and Assessment No. 121
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The test design is novel and the achieved endpoints cannot be used in an EU ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Assessment and conclusion by RMS:

A full life-cycle (240 days) bioassay using the terrestrial snail, *Cantareus aspersus*, allowing exposure during embryogenesis and/or the growth and reproduction phases, was used to assess the effects of Bypass®, a glyphosate-based herbicide, on a range of endpoints, including parameters under endocrine control.
It is clearly stated that the surfactant polyethoxylated tallow amine (POEA) was present in the formulation which is known to be more toxic than glyphosate to others non-target groups. Since August 2016, POEA is not authorized in plant protection products containing glyphosate (European Commission). Then it is not possible to discriminate between glyphosate and POEA.
Due to presence of POEA, the study is considered not relevant by RMS.

Data point:	CA 9
Report author	Ada F. B. *et al.*
Report year	2013
Report title	Ganado-hepato-somatic index of *Oreochromis niloticus* sub adults exposed to some herbicides
Document No	International Journal of Aquaculture (2013), Vol. 3, No. 11
Guidelines followed in study	Not stated
Deviations from current test guideline	Not applicable as test did not follow a recognised test guideline. No environmental conditions recorded.

	No analytical measurements to confirm exposure.
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The gonadosomatic and hepatosomatic indexes were measured after exposing the fish to different concentrations of Gramoxone, Glyphosate, Butachlor and Atrazine for fourteen days. These herbicides were observed to produce atrophy in the two organs, liver and gonads. The herbicides are therefore likely going to hinder fish production in the area due to their influence in these important fish organs.

Materials and Methods

Test substances: Glyphosate (ROUNDUP)
Paraquat (Gramoxone)
Butachlor (Rizene)
Atrazine

Exposure concentrations:	Glyphosate;	control, 0.4, 0.8, 1.2, 1.6, 2.0 and 2.4 mg/L
	Paraquat;	control, 0.9, 1.8, 2.7, 3.6, 4.5, and 5.4 mg/L
	Butachlor;	control, 0.15, 0.3, 0.45, 0.6, 0.75 and 0.9 mg/L
	Atrazine;	control, 0.8, 1.6, 2.4, 3.2, 4.0, and 4.8 mg/L

Fish species*:*	Nile Tilapia; *Oreochromis niloticus;*
Fish Size:	Between 5 g and 14 g
Fish age:	Sub-adults
Source:	Department of Fisheries and Aquatic Sciences Fish Farm
Test design:	Static test design
Test duration:	14 day exposure period
Replication:	Not stated – *assumed* single aquarium per treatment group incl. control.
Fish per replicate:	10 fish per replicate
Test volume (L):	40
Fish loading:	1.25 g fish/L to 3.5 g fish/L
Feeding:	Fed for 14 days.

Environmental conditions: Not stated.

Study Description
Individual groups of 10 fish were exposed to each active substance test concentration for 14 days and slaughtered at the end of the test. The fish, gonads and liver of fish of five fish from each exposure group were measured. The gonadosomatic and hepatosomatic index of each fish was analysed using analysis of variance (ANOVA) a 0.05% alpha level by spss Window 7 (Statistics for political and social sciences, Window 7, version 20.0) The post hoc comparison of means was carried out using Duncan's multiple range tests (Frank and Althoen, 1995).

Results

The hepatosomatic index of *Oreochromis niloticus* juveniles showed inverse relationship with Gramoxone concentration. The hepatosomatic index of control group was higher. It reduced almost steadily with concentration of Gramoxone administration (Fig. 1). The differences were significant when data were analysed using ANOVA. The Hepatosomatic Index also decreased with increase in concentration of Glyphosate (Fig. 1). Means having the same superscripts are statistically the same while those with different superscripts are statistically different $p<0.05$ using ANOVA. When comparing the weight of the liver to that of the fishes' total weight, it was revealed that the weight of the liver was reducing faster compared to that of the fishes' body weight. So, the hepatosomatic index was reducing with increase in concentration of Butachlor (Fig. 1). Similar observations were seen in fish exposed to Atrazine (Fig. 1). Similar relationships were observed in the gonads of the fish exposed to these xenobiotics. These are shown in Fig. 2 for Gramoxone, Glyphosate, Butachlor and Atrazine respectively.

Figure 1. Hepatosomatic index of *Oreochromis niloticus* sub adults exposed to different concentrations of Glyphosate, Gramoxone, Butachlor, Atrazine for 14 days. Means with different letters are significantly different.

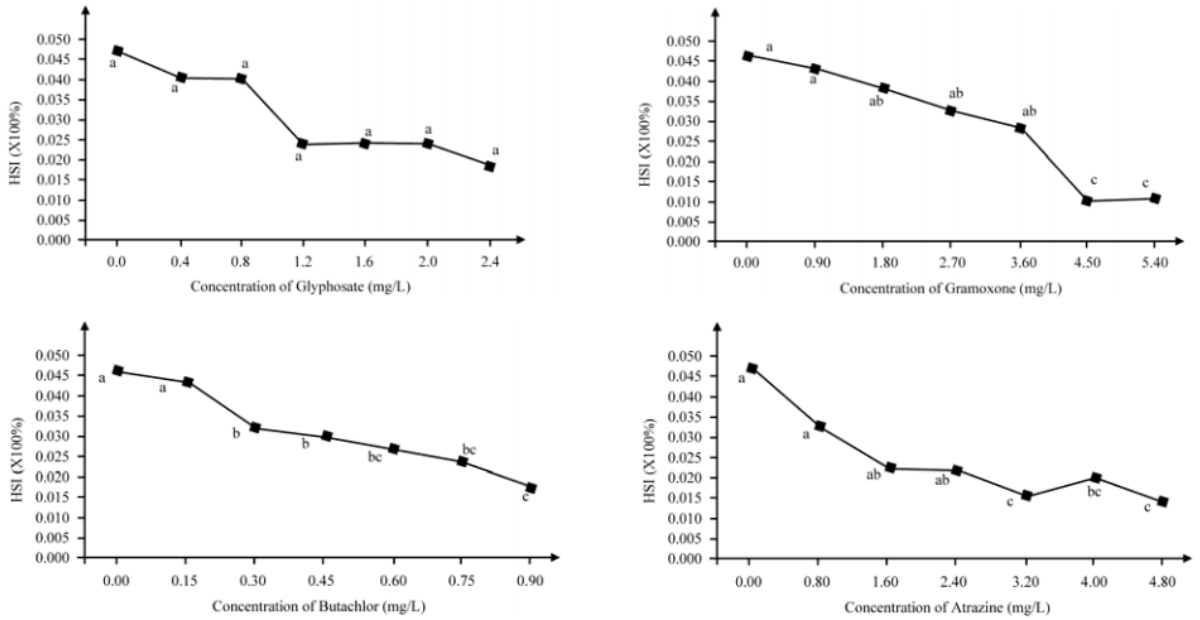
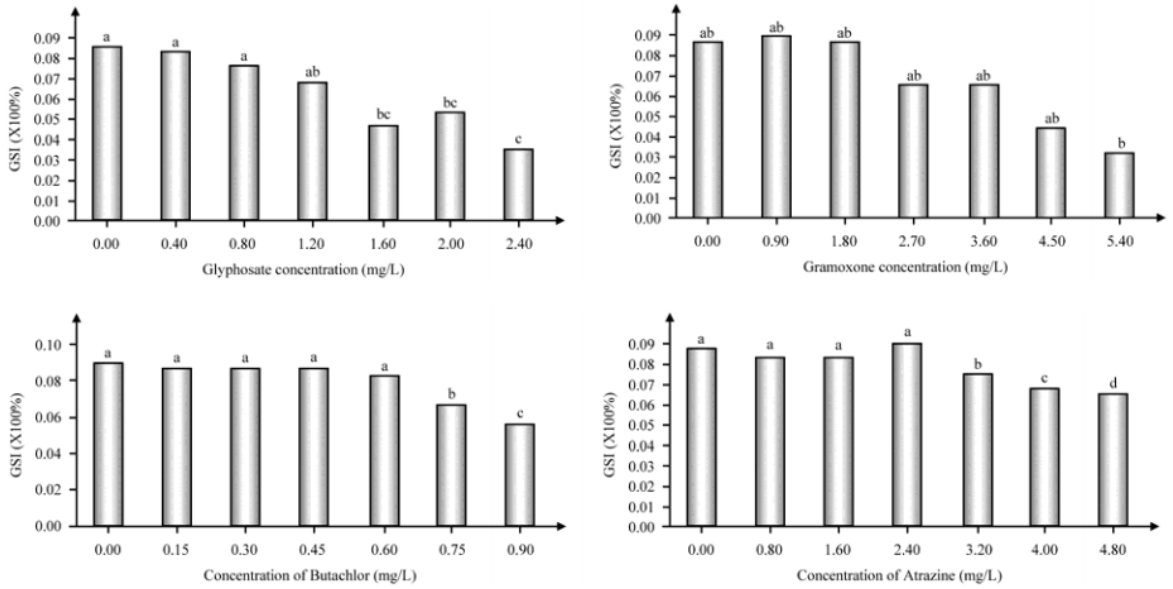


Figure 2. Gonadosomatic index of *Oreochromis niloticus* exposed to different concentrations of Glyphosate, Gramoxone, Butachlor, Atrazine for 14 days Note: Columns represent means, and means with different letters are significantly different ($p < 0.5$).



Conclusion

The gonadosomatic and hepatosomatic indexes were measured after exposing the fish to different concentrations of Gramoxone, Glyphosate, Butachlor and Atrazine for fourteen days. These herbicides were observed to produce atrophy in the two organs, liver and gonads. The herbicides are therefore likely going to hinder fish production in the area due to their influence in these important fish organs.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Endpoints based on gonadosomatic and hepatosomatic indeces are not used in the EU level ecotoxicological risk assessment and therefore the article is not relevant for the glyphosate EU renewal.

Further points of clarification:
In addition, it is not possible to confirm the identity of the glyphosate test substance used in the study as only Roundup is mentioned. Roundup is a brand that contains multiple glyphosate-based herbicides, that contain different co-formulants. Of most importance to the toxicity profile associated with a particular product is whether that product contained a polyoxyethyleneamine (POEA) surfactant which is not permitted for use in the EU. Given the country in which the study was conducted and the age of the paper, the study may have been conducted using Roundup Original, which contains POEA. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The gonadosomatic and hepatosomatic indexes were measured after exposing the Nile Tilapia (Oreochromis niloticus) to Glyphosate (in Roundup) for 14 days.
No information is available on the surfactants in the formulation that was used. Effects were observed but it is not possible to discriminate between glyphosate and surfactants. Atrophy in the two organs, liver and gonads were observed.
No specific guideline was followed. No analytical verification available. Environmental conditions were not reported. Only graphs are presented. Statistics are unclear (absence of significance despite obvious decrease on hepatosomatic index).
Overall the study is considered less relevant but supplementary (due to different formulation tested) and not reliable by RMS.

Data point:	CA 9
Report author	Avigliano L. *et al.*
Report year	2014
Report title	Effects of glyphosate on egg incubation, larvae hatching, and ovarian rematuration in the estuarine crab *Neohelice granulata*
Document No	Environmental Toxicology and Chemistry (2014), Vol. 33, no. 8, pp. 1879
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

Ovigerous females of the estuarine crab (*Neohelice granulate*) were exposed to both pure glyphosate (2.5 mg/L and 5 mg/L) and a glyphosate formulation (Roundup Ultramax, containing glyphosate at 2.5 mg/L acid equivalent). At the end of the egg incubation period, a significant reduction in the number of hatched larvae was the result of Roundup Ultramax exposure. Additionally, several larvae abnormalities were seen in both pure glyphosate (2.5 mg/L) and Roundup Ultramax treatments, such as hydropsy and hypopigmented eyes, and atrophied eyes were observed in the Roundup Ultramax treatment. To evaluate the effect of the herbicide on ovarian rematuration, females remained exposed for 32 d. Pure glyphosate at 2.5 mg/L stimulated ovarian maturation over control levels, mainly in terms of a higher gonadosomatic index and a higher percentage of vitellogenic oocytes. A plausible hypothesis to be tested in further experiments is that exposure to glyphosate disrupts the hormonal system controlling reproduction.

Materials and methods

Ovigerous N. granulata females carrying immature eggs were randomly collected (13 October 2012) at the southern edge of Samborombón Bay, a nonpolluted area at the mouth of the Rio de la Plata estuary, Argentina. Once in the laboratory, they were acclimated for 12 h at the same environmental conditions and water quality used later for bioassays. All bioassays were conducted in semi-static conditions according to the standard procedures recommended by the American Public Health Association. Stock solutions of glyphosate (as acid, 99.8% purity; Sigma) and Roundup Ultramax (as soluble granules, 67.9% w/w of glyphosate a.e.; Monsanto) were prepared weekly by dissolving the appropriate amount of the chemicals in distilled water. Small aliquots from these stock solutions were added to the test recipients with the appropriate dilution water, as stated below.
The nominal concentrations assayed for pure glyphosate were 2.5 mg/L and 5 mg/L, whereas 2.5 mg/L was used for the Roundup Ultramax treatment. These sublethal concentrations were chosen according to the results of a preliminary range finding test, carried out according to the guidelines given by the American Public Health Association. Prior to the beginning of the assay, replicated water samples (15 mL) were taken at 0 h and 72 h, the period for water replacement in all test containers, to validate nominal concentrations. Glyphosate concentration was determined with ion-exchange chromatography, using a DionexDX-100 chromatograph with a conductivity detector and a 25-mL sample loop. A Dionex AS-4 was used as an analytical chromatographic column with an experimental error below 5%. A mixture of $NaOH/Na_2CO_3$ 4 mM/9mM was chosen as eluent with a flow rate of 2 mL/min.

Effects on hatching
Eleven ovigerous females were randomly assigned to each treatment and control, and each female was placed in a glass container filled with 500 mL of saline water prepared by diluting artificial seawater salts (Tetra Marine Salt Pro) in dechlorinated tap water (hardness 80 mg/L as equivalents of $CaCO_3$, final salinity 30‰, pH 8.0 ± 0.5) provided with constant aeration. The solution of each container was completely replaced every 72 h. Females were not fed during the assay, and they were checked daily for mortality, egg loss, and hatching larvae. A temperature of 25 ± 18°C and a photoperiod of 14:10 h (light:dark) were maintained throughout. The exposure period comprised an overall mean of 9.86 ± 0.57 d (n = 44), from the beginning of the assay until larval hatching. After hatching, 2 samples of 10 mL each were taken from each container, stirred to homogenize larvae distribution, and fixed in 5% formalin. The total estimated mean number of hatched larvae was calculated from 2 random samples taken in relation to the total volume of water in the container. To determine the proportion of morphological abnormalities in each spawning, a random subsample of 50 larvae was examined under a stereomicroscope, and the incidence of each abnormality detected was then registered.

Effects on ovarian rematuration
Post-hatching, females remained exposed to the same treatments until day 32, counting from the beginning of the assay. They were maintained under the same experimental conditions described above but fed pellets prepared in the laboratory (an amount equivalent to 5% of body mass, twice per week), supplemented with *Elodea* sp. fresh leaves *ad libitum*. At the end of the assay, females were weighed to determine body weight, and both the ovaries and the hepatopancreas were dissected and weighed to determine the gonadosomatic (GSI) and hepatosomatic (HSI) indexes as GSI or HIS = (gonad wet wt or hepatopancreas wet wt/body wt)x100. Ovaries were weighed and fixed in Bouin solution for 4 h at room temperature, dehydrated in alcohol series, and finally embedded in paraplast. Then, 5-mm sections were prepared and stained with hematoxylin and eosin. For each animal, a representative section of the ovary was analyzed to determine both the relative proportions of normal and abnormal oocytes and oocyte area. Previtellogenic, intermediate, and vitellogenic oocytes were characterized according to their size and degree of basophilia. To assess the proportions of normal and abnormal oocytes, a grid of 100 points, mounted on an 8x ocular lens, was used in combination with a 40x objective lens. At least 3 ovarian sections from each animal were examined by this procedure. For oocyte area, both major and minor diameters of the oocytes showing their nuclei were estimated by means of a micrometric ocular lens, calibrated against a Leitz-Wetzlar plate with 1/100 mm spacing, to calculate the oocyte area as (π/4) x major diameter x minor diameter.

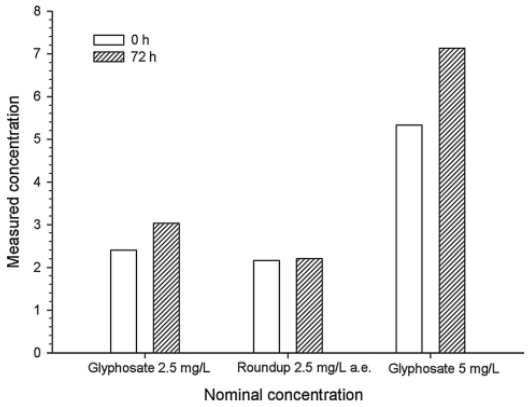
Statistical analysis

A one-way analysis of variance followed by least significant difference multiple comparisons was used for testing differences between experimental groups concerning incubation time, number of hatched larvae, proportion of each abnormality, proportion of each oocyte type, and oocyte area. Logarithmic or angular transformation of data was used when homogeneity of variances was not confirmed in raw data by the Bartlett test. Proportions of both surviving females and females with egg loss were compared between experimental groups by the Fisher exact test.

Results

Measured concentrations of both pure glyphosate and Roundup Ultramax were relatively close to the nominal concentration (r^2 = 0.988 and 0.954 for 0 h and 72 h, respectively; Fig. 1).

Figure 1. Nominal concentrations versus concentrations measured at 0 h and 72 h of the water replacement period.



Effects on hatching

Ovigerous females exposed to glyphosate did not experience mortality during the egg incubation period. In addition, no significant ($p>0.05$) differences were observed among mean body weight of females (overall mean 9.85±0.43 g). Because no loss of eggs was verified in any case, 100% of females had normal hatching. No significant differences ($p>0.05$) were observed in incubation time, although a significant ($p<0.05$) reduction in the number of hatched larvae per female was observed in the Roundup Ultramax treatment compared with control (Table 1).

Table 1. Percentage of hatching females, incubation time, and number of hatched larvae per female for each treatment

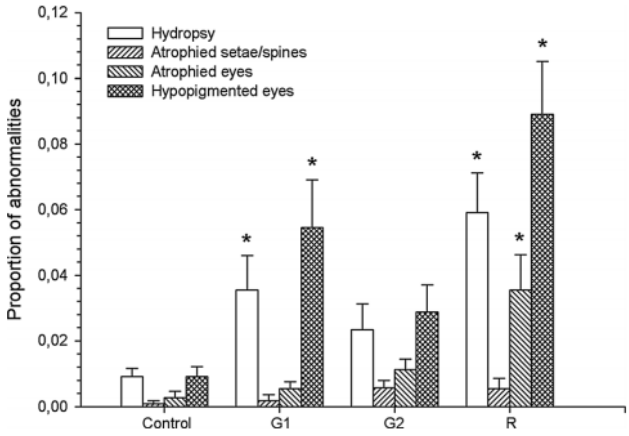
Glyphosate nominal concentration (mg/L a.e.)	No. of females	Hatching females (%)	Incubation time (d)[a]	No. of hatched larvae per female[a]
Control	11	100.00	9.72 ± 1.28	33 745 ± 4969
Glyphosate, 2.5	11	100.00	11.27 ± 1.34	26 739 ± 5519
Glyphosate, 5	11	100.00	9.18 ± 0.76	25 472 ± 4472
Roundup, 2.5	11	100.00	9.27 ± 1.11	15 448 ± 2036*

[a]Mean ± standard error.
*Significant difference ($p < 0.05$) with respect to control.
a.e. = acid equivalent.

Morphological abnormalities observed in larvae hatched from ovigerous females exposed to glyphosate during the egg incubation period were as follows: hydropsy, related to an abnormal hydration of tissues, especially in the cephalotorax; atrophy of spines and setae, probably underdeveloped, especially in the maxillipeds; atrophy of eyes, which showed an abnormal contour; and hypopigmented eyes, because of loss of screening pigments in ommatidia. The incidence of these abnormalities is shown in Fig. 2. Compared with the control group, a significantly ($p<0.05$) higher incidence of both hydropsy and hypopigmented eyes was observed for both glyphosate and Roundup Ultramax groups (2.5 mg/L of glyphosate a.e. in both cases). Moreover, a higher incidence of atrophied eyes was observed in the Roundup Ultramax

treatment.

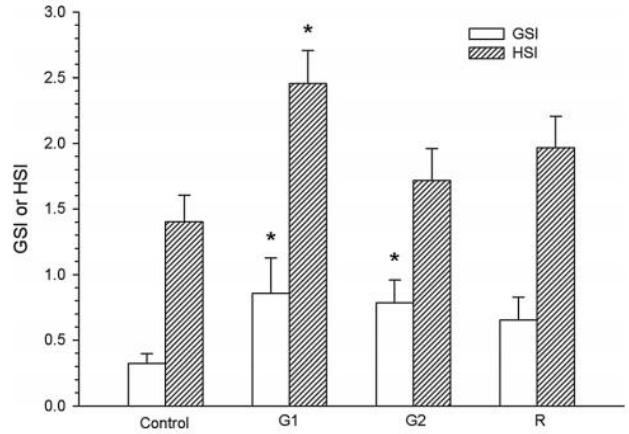
Figure 2. Morphological abnormalities in larvae hatched from ovigerous females exposed to either glyphosate (G) or Roundup Ultramax (R). Mean ± standard errors are indicated. G1=2.5 mg/L acid equivalent (a.e.); G2=5 mg/L a.e.; R= 2.5 mg/L a.e. *Significant difference ($p<0.05$) with respect to control.



Effects on ovarian rematuration

No significant mortality of females exposed to glyphosate during the 32 d of the ovarian rematuration assay was seen when compared with the control group, for which mortality reached 27% at the end of the assay. Both GSI and HSI, determined at the end of the 32-d rematuration assay, are shown in Fig. 3. Whereas a higher HIS was observed only for the lower glyphosate concentration compared with the control, a significantly higher GSI value was observed in the group exposed to pure glyphosate at both concentrations assayed. No significant ($p>0.05$) differences were seen in the area of any type of normal oocytes whose mean area values were 1158.62 ± 42.36 μm^2, 4013.50 ± 286.98 μm^2, and 10 322.87 ± 1614.91 μm^2 for previtellogenic, intermediate, and vitellogenic oocytes, respectively. Significant ($p<0.05$) differences in the percentage of normal oocytes were only found between control and glyphosate at 2.5 mg/L, indicating a lower percentage of intermediate and a higher percentage of vitellogenic oocytes with exposure to the herbicide. Although glyphosate increased the percentage of reabsorbed oocytes (particularly the vitellogenic ones in Roundup Ultramax treatment, Fig. 3), no significant ($p>0.05$) differences with respect to control were detected.

Figure 3. Gonadosomatic index (GSI) and hepatosomatic index (HSI) for each treatment at the end of the 32-d assay. G1=2.5 mg/L acid equivalent (a.e.); G2=5 mg/L a.e.; R=2.5 mg/L a.e. Mean ± standard errors are indicated. *Significant difference ($p<0.05$) with respect to control.



Conclusion

At the end of the egg incubation period, a significant reduction in the number of hatched larvae was the result of Roundup Ultramax exposure. Additionally, several larvae abnormalities were seen in both pure glyphosate (2.5 mg/L) and Roundup Ultramax treatments, such as hydropsy and hypopigmented eyes, and atrophied eyes were observed in the Roundup Ultramax treatment. Pure glyphosate at 2.5 mg/L stimulated ovarian maturation over control levels, mainly in terms of a higher gonadosomatic index and a higher percentage of vitellogenic oocytes.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: An Argentinian study, which can be difficult to extrapolate to EU (e.g. local native species, geo-climatic properties, land-uses and agricultural practices differ from EU). The article discusses effects of pure glyphosate and Roundup Ultramax on crab development. Difficult to relate findings of the study to an EU level ecotoxicological risk assessment as they are based on GSI and HIS values. In addition, the formulation tested, Roundup Ultramax (67.9% w/w of glyphosate a.e.; formulated using potassium salt) is not the representative formulation for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

This study was part of the previous EU assessment for endocrine disruption (2017).

Ovigerous females of the estuarine crab (Neohelice granulate) were exposed to both pure glyphosate (2.5 mg/L and 5 mg/L) and a glyphosate formulation (Roundup Ultramax, containing glyphosate at 2.5 mg/L acid equivalent). This study investigated effects on developing embryos, hatched larvae, and ovarian rematuration of N. granulata during the reproductive period. The study did not follow standardized guideline.

The nominal concentrations used for pure glyphosate were 2.5 mg/L and 5 mg/L, whereas 2.5 mg/L was used for the Roundup Ultramax treatment. Analytical verifications of tested concentrations have been made.

No mortality or effect on bodyweight was observed on ovigerous females. Because no loss of eggs was verified in any case, 100% of females had normal hatching. A significant reduction in the number of hatched larvae per female was observed in the Roundup treatment. (note: exposure period till hatching 9.86 d). A significantly higher incidence of both hydropsy and hypopigmented eyes was observed for both glyphosate and Roundup groups (2.5 mg/L of glyphosate a.e. in both cases). Pure glyphosate at 2.5 mg/L stimulated ovarian maturation, mainly in terms of a higher gonadosomatic index and a higher percentage of vitellogenic oocytes (after 32 days exposure).

A significantly lower number of hatched larvae per female was detected in the Roundup Ultramax treatment, a clear embryonic mortality was associated with this formulation, which contained a glyphosate concentration of 2.5 mg/L. Also taking into account that pure glyphosate, at the same concentration, did not significantly reduce the number of hatched larvae, these results indicate that Roundup compounds other than glyphosate may be responsible for the embryonic mortality.

The results available for the pure form of glyphosate are deemed relevant by RMS (i.e. stimulated ovarian maturation at 2.5 mg/L, mainly in terms of a higher gonadosomatic index and a higher percentage of vitellogenic oocytes).

This study shows effects potentially related to endocrine disruption. In accordance with the EFSA guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, such effects (including malformations) should be considered.
Relevant for the assessment of potential for endocrine disruption.

The results of the range-finding study were not presented. Then the MTC cannot be determined.
Concerning the potential impact of using wild-caught organisms, particularly regarding potential effects on the endocrine system from prior exposure to other substances, it is mentioned in the report that the specimens were randomly collected at the southern edge of Samborombón Bay, a "nonpolluted" area at the mouth of the Rio de la Plata estuary, Argentina. The contamination level cannot be verified by RMS, it is only stated that very scarce information about glyphosate environmental levels has been published, with reported values between 0.1 mg/L and 0.7 mg/L in water and between 0.5 mg/kg and 5mg/kg in sediments. The study authors then assume the tested concentrations are higher than the overall levels of glyphosate found in Argentina but still relevant for a direct run-off entry and comparable to levels measured in other countries. Besides, Avigliano et al, 2018 (also assessed by RMS) states that "*heavy charge of herbicides and other pesticides is carried by several rivers and channels that cross extensive agricultural areas and finally reach the Samborombón Bay (Comisión Administrativa del Río de La Plata 1990)*". RMS then cannot discard the presence of other toxicants in this estuary.

Control mortality was relatively high at the end of the assay (27% at 32 days) although RMS acknowledges that data on "natural" mortality is lacking. Mortality averaged 9% in the glyphosate treatments, which did not differ from control.
Regarding the morphology of the larvae, statistically significant effects were observed in the treatment with lower glyphosate, but not in the treatment with higher glyphosate concentrations. In the glyphosate treatments, data showed high variability, which likely hampered the detection of statistically significant results. The observed increases in gonadosomatic index (without concurrent hepatosomatic index increases) are likely due, as the authors supposed, to increased egg resorption, but the reason or the specific mechanism for these observation remains unclear and might be due to general toxicity. It is noted that the effects on morphology did not follow a dose-response relationship in the treatments with glyphosate.

Overall, it is not possible to relate the observed effects to an endocrine mode of action.

The study is relevant but results considered unreliable for ED assessment.

Data point:	CA 9
Report author	Reddy S. B. *et al.*
Report year	2018
Report title	Disturbances in reproduction and expression of steroidogenic enzymes in aquatic invertebrates exposed to components of the herbicide Roundup
Document No	Toxicology Research and Application (2018), Vol. 2, pp. 2397847318805276/1
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

The researchers have investigated the effects of the individual active ingredients of the herbicide Roundup (glyphosate and diquat dibromide [DD]) since Roundup causes alterations in reproduction, mortality, and development in the aquatic snail *Lymnaea palustris*. Snails chronically treated with elevated but ecologically relevant levels of DD exhibit reduction in fecundity ($p < 0.05$), while fecundity in glyphosate treated snails is comparable to or exceeds control levels. To investigate a possible mechanism for the reproductive disturbance, the researchers monitored levels of steroid acute regulatory (StAR) protein in whole snails and observed a correlation in StAR protein decrease with treatment with Roundup, glyphosate, or DD. The researchers detect StAR in organs where steroid biosynthesis occurs

(ovotestis, brain, kidney); StAR protein is reduced following chronic exposure to Roundup, glyphosate, or DD ($p < 0.01$). Estradiol and testosterone concentrations in hemolymph were measured by enzyme-linked immunosorbent assay following 3-week exposure of snails to 3.5 mg/L glyphosate or 140 µg/L DD. Testosterone levels decrease in DD-treated groups ($p < 0.05$); a trend of lower testosterone is also observed in glyphosate-treated groups ($p > 0.05$). Estradiol concentration is greater than or equal to control levels in glyphosate but decreased in DD ($p < 0.05$). Because of its role in the conversion of testosterone to estradiol, the researchers monitored abundance of aromatase and observed a reduction ($p < 0.05$) in DD-treated snails (consistent with the drop in fecundity and estradiol levels) and a comparable level to control in glyphosate-treated snails (consistent with their high fecundity and estradiol levels). Although the toxicity of commercially available Roundup to aquatic animals may have many contributing factors including its inactive surfactant, the constituent of Roundup associated with the greatest reproductive disturbances and observed developmental abnormalities of offspring is DD. This study details the analysis of particular herbicide constituents and their effect on specific targets in the reproductive pathway.

Materials and methods

Animal culture and treatment

Laboratory-reared *L. palustris* were housed in filtered aerated aquaria in artificial pond water (APW) and fed rinsed organic romaine lettuce ad libitum. Adult snails used in the study ranged from 1.4 cm to 1.8 cm in shell height; 400 mL covered mesocosms with aeration were used for all adult chronic treatments; snails were housed at four adults per unit on a 12:12 light-dark cycle at 21 ± 1°C. Mesocosms were maintained with 100% change of solution twice weekly. Jelly masses containing fertilized eggs are oviposited by adults on tank walls or bottom, then harvested and the total number of embryos per tank recorded twice weekly. Three mesocosms were established for each of three treatment types (n = 36) and housed for 3 weeks: control APW, DD (140 µg/L), and glyphosate (3.5 mg/L), and repeated for a total n = 72. Other animals in parallel and pilot studies (n = 108) were established similarly during which snails were housed for up to 6 weeks; although guidelines are in place for 4-week chronic exposure times of related species, 3 weeks of exposure is also common in pond snails and consistently yields reproductive disturbance in our species. Animals were exposed to complete Roundup at 19.5 mg/L; the concentrations of DD and glyphosate solutions were set at five times the US EPA MCL (glyphosate) or MCLG (DD), or allowable concentration in drinking water (EPA 2018), and the Roundup concentration was based on five times the MCL of glyphosate. These concentrations, while in excess of human drinking water recommendations, are typically exceeded in surface waters for a period of days or longer following application of DD or glyphosate-containing herbicides.

Steroid analysis

Hemolymph was drawn weekly (once at outset of study, and following each of 3 weeks of treatment) from snails using a noninvasive, nonlethal method whereby the animal is induced to extrude hemolymph by a poke on the foot with a pipette tip. Hemolymph was flash frozen in liquid nitrogen and stored at -80°Cuntil analysis by enzyme-linked immunosorbent assay (ELISA). Hemolymph, approximately 50 µL per animal per draw, was pooled from all individuals in one mesocosm at a given time point. ELISA analyses for estradiol and testosterone were conducted and data analyzed according to manufacturer's instructions. Absorbance of samples and standards was read in BioTek, Winooski, VT Synergy HT Multi-Detection Microplate Reader and calculations made in Microsoft Excel ($r^2 > 0.995$).

Protein analysis

To analyze differential aromatase abundance, ovotestes and associated structures (prostate and albumen glands) were harvested at the end of the study period. Tissues were mechanically disrupted in RIPA buffer (Thermo Fisher, Waltham, MA 89900) containing protease inhibitor cocktail (Thermo Fisher 78425) according to manufacturer's directions, and snap frozen in liquid nitrogen followed by storage at -80°C prior to analysis by Western blot. Samples were O- and N-glycosidased, approximately 1-10 µg per sample (at fixed volume) run under denaturing conditions on 12% polyacrylamide gel electrophoresis, and transferred using iBlot 2 Gel Transfer System (ThermoFisher). Glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) was used as a housekeeping protein (Rockland Immunochemicals, Limerick, PA rabbit polyclonal anti-GAPDH primary antibody, 1:1000) for relative aromatase abundance calculation (ThermoFisher PA1-21398 rabbit polyclonal anti-aromatase primary antibody, 1:100). Following secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit; GE Healthcare Bio-Sciences, Pittsburgh, PA NA934, 1:10,000) and application of Super Signal West Pico PLUS Chemiluminescent Substrate (ThermoFisher 34577), bands were imaged on a Bio-Rad ChemiDoc-MP and quantified using Image Lab software (Bio-Rad Hercules, CA).
To analyze StAR abundance in whole snail, ovotestis, kidney, or brain, tissues were isolated and disrupted similarly using ReadyPrep Protein Extraction Kit (Bio-Rad 163-2086). Samples were quantified using EZQ Protein Quantitation Kit (Invitrogen Carlsbad, CA/ThermoFisher R33200), and 30 µg of total protein or 10 µg of individual organ protein was loaded in each lane for 12% PAGE and transfer as above. Primary anti-StAR at 1:1000 and subsequent secondary, treatment, and analysis as described above to quantify relative intensity of bands in control and treated samples.

Statistical analysis
Paired t-tests comparing glyphosate and DD treatment groups relative to controls were conducted on embryo counts (fecundity), hemolymph concentrations of estradiol and testosterone, and relative abundance of StAR protein present in control, glyphosate-, and DD-treated samples. Normalized abundance of aromatase protein present in control, glyphosate-, and DD-treated samples were compared by X^2 analysis using the control levels as the expected range.

Results

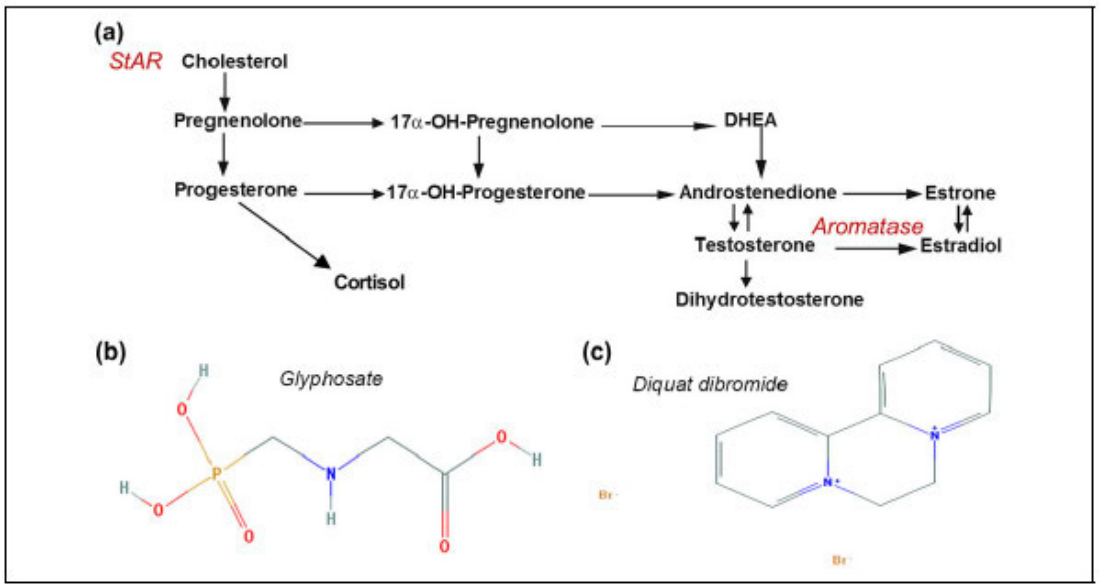


Figure 1. (a) Steroidogenic pathway in molluscs. StAR is the rate-limiting factor in steroidogenesis and ushers cholesterol to the inner mitochondrial membrane. P450-aromatase (CYP19 or aromatase) converts testosterone to estradiol. Panel adapted from Janer and Porte.[28] (b and c) Chemical structures of herbicides used in this study, generated by National Center for Biotechnology Information PubChem Compound Database: (b) structure of glyphosate and (c) structure of diquat dibromide. StAR: steroid acute regulatory protein.

Fecundity
Embryo counts collected from each mesocosm were normalized by the number of snails alive at that time point to ensure accurate representation of the fecundity per individual for each treatment type over the 3-week study, since a low level of spontaneous mortality occurred. As the study progressed, DD-treated snails exhibited a marked decrease in embryo production (*$p < 0.05$). The fecundity of glyphosate-treated snails did not differ significantly from the control animals over the course of the study, and at several time points exceeded control fecundity. To determine the cause of the disruption in fecundity, steroid sex hormone levels and steroidogenic pathway component abundance were

analyzed.

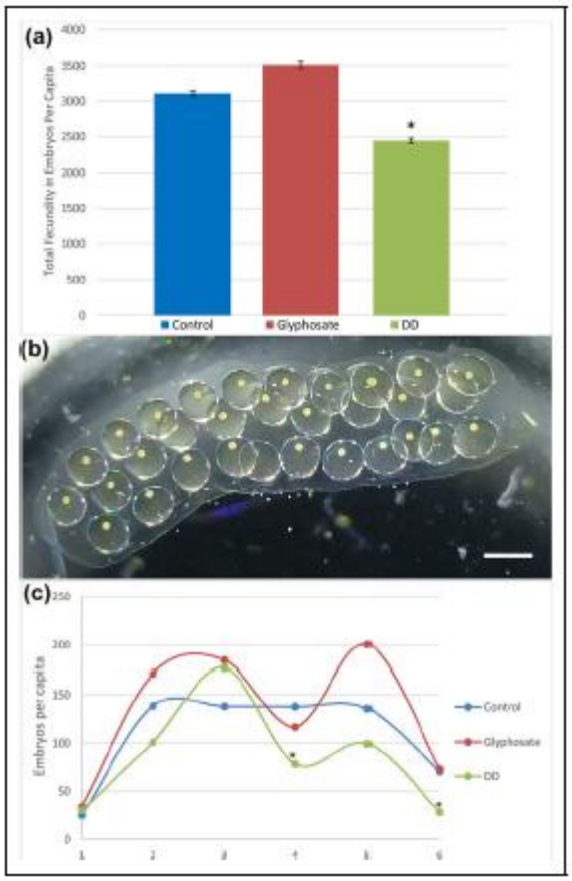


Figure 2. Fecundity of *L palustris* following treatment with glyphosate or DD. (a) Total fecundity per snail in control APW, 3.5 mg/L glyphosate treatment, and 140 µg/L DD treatment over 3-week study. Twice weekly harvested embryos were normalized to number of living individuals to obtain a per capita count. The six per capita counts were averaged and fecundity in each treatment was compared to control fecundity using two-tailed *t*-test; *$p < 0.05$. Error bars represent SEM. (b) Jelly mass of *L palustris*; bar = 1 mm. (c) Average fecundity per capita in each treatment over the course of the study was assessed twice weekly with embryo counts per live individual numbered sequentially 1–6. Embryo counts at each time point in each treatment were compared to control embryo counts using two-tailed *t*-test; *$p < 0.05$. DD: diquat dibromide; APW: artificial pond water; SEM: standard error of the mean.

Steroid hormone levels

Hemolymph extracted from animals prior to the outset of the study and weekly for 3 weeks during treatment was analyzed by ELISA for estradiol and testosterone concentrations. In glyphosate-treated animals, a consistent trend of estradiol concentration comparable to or exceeding that of control animals ws observed. Testosterone levels in glyphosate-treated animals are statistically comparable to control animals ($p = 0.06$), although consistently lower than controls and in decreasing concentration relative to control animals as the study progressed beyond the 1-week mark. Hemolymph of animals treated with DD contains significantly lower estradiol and testosterone concentrations relative to control animals. The differences were most pronounced at the 3-week mark, when estradiol and testosterone concentrations in DD treated animals are lowest relative to controls (**$p < 0.01$).

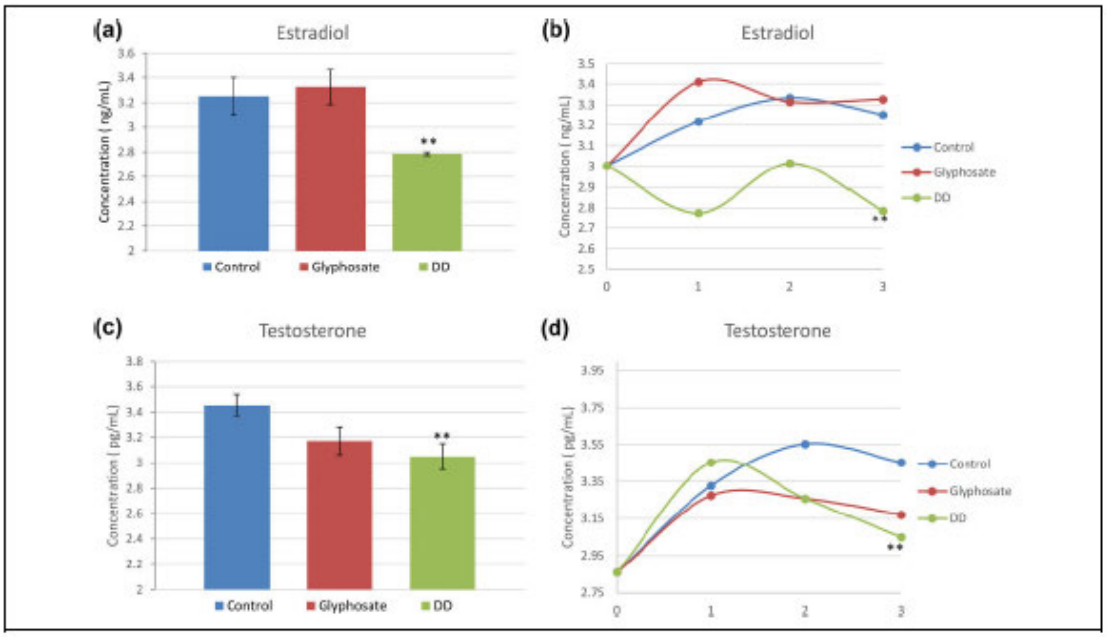
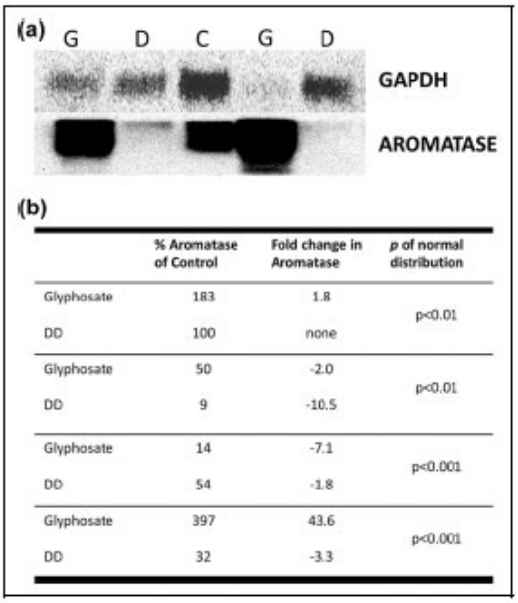


Figure 3. Testosterone and estradiol levels measured by ELISA in *L. palustris* hemolymph throughout 3-week exposure to glyphosate or DD. (a) Hemolymph estradiol concentration in ng/mL at the conclusion of the study. (b) Graph of fluctuations in estradiol concentration over course of weekly hemolymph draw and analysis; zero represents the draw prior to the outset of treatment and each number represents the draw at the conclusion of each week. (c) Hemolymph testosterone concentration in pg/mL at the conclusion of the study. (d) Graph of fluctuations in testosterone concentration over course of weekly hemolymph draw and analysis; zero represents the draw prior to the outset of treatment and each number represents the draw at the conclusion of each week. Error bars represent SEM; **$p < 0.01$. DD: diquat dibromide; ELISA: enzyme-linked immunosorbent assay; SEM: standard error of the mean.

Western blot analyses

Western blot analysis on isolated ovotestes from the various treatment groups at the conclusion of the 3-week study revealed that glyphosate-treated samples on average had a larger quantity of aromatase protein compared to control samples, and DD-treated samples had a lower quantity of aromatase protein compared to control samples. Although some DD-treated ovotestis samples had comparable aromatase or approximately two- to three-fold less than control samples, most had little to no detectable aromatase, although GAPDH detection reveals there was equivalent protein present in the sample. Ovotestes from glyphosate-treated animals contained the highest levels of aromatase protein across all samples analyzed (n = 44); however, there was no significant difference in aromatase level between control and glyphosate-treated samples, and glyphosate-treated samples in particular exhibited very high variability between individuals. When analyzed in sets corresponding to separate Western blots (each set containing DD-treated, glyphosate-treated, and control individuals' ovotestis), the distribution of aromatase content in DD and glyphosate samples was found to differ greatly ($p < 0.01$) from the normal range of distribution found in control samples.

StAR protein is readily detected in snail brain, kidney, and ovotestes, but is not detected in other snail organs tested (lung, heart). Following 6-week chronic treatment in Roundup, DD, or glyphosate, whole snails as well as isolated kidney, brain, and ovotestes were analyzed to quantify decrease in StAR expression. Glyphosate-treated whole animal samples exhibited a 42% reduction in StAR, Roundup-treated samples on average exhibited a 65% reduction in StAR, and DD-treated whole animal samples exhibited a 70% reduction in StAR, suggesting that both active ingredients of Roundup may contribute to the downregulation of StAR in the whole animal when chronically exposed. When individual organs from chronically treated animals were analyzed, the organs were affected differentially by the same treatments. The kidney exhibited a less than two-fold reduction in StAR following 6-week treatment with Roundup (62% of control) or DD (55% of control), while the brain exhibited greater than four-fold reduction in StAR following 6-week treatment with Roundup (22% of control) or DD (23% of control), and the ovotestis was the steroidogenic organ most affected by Roundup (6% of control) or DD (9% of control) with greater than 13-fold reduction in StAR.



(b)

	% Aromatase of Control	Fold change in Aromatase	*p* of normal distribution
Glyphosate	183	1.8	p<0.01
DD	100	none	
Glyphosate	50	-2.0	p<0.01
DD	9	-10.5	
Glyphosate	14	-7.1	p<0.001
DD	54	-1.8	
Glyphosate	397	43.6	p<0.001
DD	32	-3.3	

Figure 4. (a) Representative Western blot of ovotestes harvested from animals exposed to 3-week treatment in control APW, glyphosate (3.5 mg/L), or DD (140 µg/L). Top: Western blot of GAPDH for internal standardization of samples. Bottom: Western blot of aromatase (G: glyphosate; D: DD; C: control APW). (b) Percent aromatase quantity in treated ovotestes relative to aromatase quantity in control ovotestes for four Western blots, demonstrating high variability of aromatase quantity in glyphosate-treated samples and consistent though variable decrease in aromatase quantity in DD-treated samples. Aromatase quantity in treated ovotestes relative to aromatase quantity in control ovotestes is also represented as fold change in aromatase. χ^2 analysis conducted on each individual blot comparing average quantity of aromatase in each treatment group relative to average quantity of aromatase in control group; treatment group aromatase levels varied significantly from expected normal distribution based on control group for each blot. APW: artificial pond water; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DD: diquat dibromide.

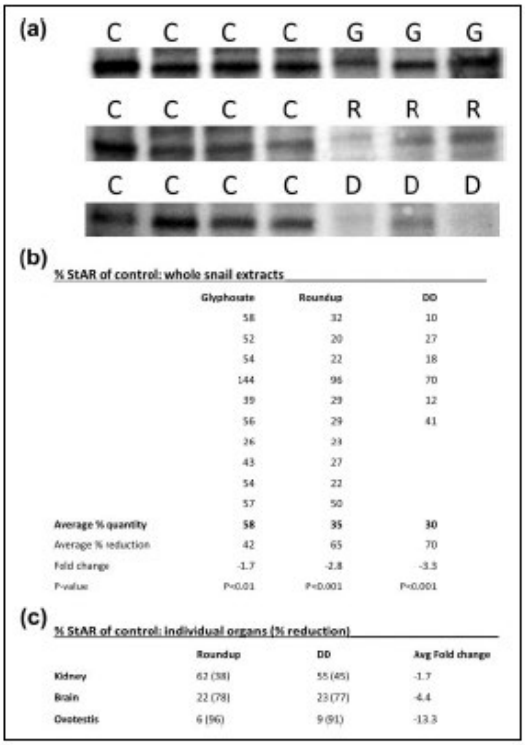


Figure 5. (a) Representative Western blots for StAR of whole snails exposed for 6 weeks to control (APW) conditions or supplemented with Roundup (19.5 mg/L), glyphosate (3.5 mg/L), or DD (140 µg/L). Representative samples from 12 control snails, 3 glyphosate-, 3 Roundup-, and 3 DD-treated snails are shown (30 µg protein total loaded per lane). C: control; G: glyphosate; R: Roundup; D: diquat dibromide. (b) Percent StAR quantity in treated whole snail extracts relative to StAR quantity in control whole snail extracts; all treated individuals except one in glyphosate exhibited a reduction in StAR, for an average reduction in StAR quantity of 41.7% for glyphosate, 65.0% for Roundup, and 70.3% for DD; average fold change in StAR in each treatment group is also shown. Each treatment resulted in significant reduction in StAR protein compared to control whole snail StAR content by *t*-test. (c) Percent StAR protein in individual organs harvested from treated snails relative to organs harvested from control snails. StAR was reduced by >90% in the ovotestis over the course of chronic DD or Roundup treatment; StAR was reduced by 77–78% in the brain and 38–45% in the kidney under the same conditions. Average fold change in StAR for Roundup and DD treatment is shown for each organ. StAR: steroid acute regulatory protein; APW: artificial pond water; DD: diquat dibromide.

A summary of the individual effects of glyphosate and DD in the system is also included.

Table 1. Trends in reproductive indicators exhibited overall in snails exposed to glyphosate or DD relative to control snails.

	Glyphosate	DD
Fecundity	↑	↓[a]
Testosterone	↓	↓[a]
Estradiol	↑	↓[a]
E:T[b]	1.03	0.93
Aromatase	↑	↓
StAR	↓[a]	↓[a]

StAR: steroid acute regulatory protein; DD: diquat dibromide; ET: estradiol to testosterone ratio.
[a]$p < 0.05$.
[b]The control E:T value is 0.95.

While glyphosate is associated with a transient rise in fecundity, a trend toward decreased testosterone and increased estradiol and fecundity leading to a modest increase in estradiol to testosterone (E:T) ratio, and potential elevation of aromatase level, the main impact of 3-week glyphosate treatment is a reduction in StAR level. In contrast, the effects of DD treatment include significant decreases in StAR, fecundity, testosterone, and estradiol leading to a modest reduction in E:T ratio, and a trend toward lower aromatase levels.

Conclusion

No conclusion was provided by the authors in the respective article.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Findings cannot be related to an EU level ecotoxicology risk assessment, as the methods used are not recognised at the EU level. In addition, the test substance used was not identified appropriately in the methods section, beyond stating '*Animals were exposed to complete Roundup at 19.5 mg/L.*' However, in the introduction, preliminary investigations of the authors invertebrate model *Lymnaea palustris* to Roundup or its individual constituents, suggests the product tested was an original formulation of Roundup that contains POEA (polyethoxylated tallowamine) surfactant, which is not permitted in agrochemical products in the EU. As the performance / efficacy of herbicidal formulations is dependant on the surfactant system / co-formulants, the findings in the paper cannot be related to the representative formulation, and are therefore not relevant to the regulatory risk assessment for the glyphosate EU renewal.

Assessment and conclusion by RMS:

Effects of the individual active ingredients of the herbicide Roundup (glyphosate and diquat dibromide [DD]) were investigated in the aquatic snail Lymnaea palustris.
Glyphosate was tested at 3.5 mg/L:

- Fecundity in glyphosate treated snails is comparable to or exceeds control levels.
- Levels of steroid acute regulatory (StAR) protein in whole snails decrease with treatment with Roundup, glyphosate, or DD.
- StAR in organs where steroid biosynthesis occurs (ovotestis, brain, kidney) reduced following chronic exposure to Roundup, glyphosate, or DD ($p < 0.01$).
- Testosterone levels decrease in DD-treated groups ($p < 0.05$); a trend of lower testosterone is also observed in glyphosate-treated groups ($p > 0.05$).
- Estradiol concentration is greater than or equal to control levels in glyphosate but decreased in DD ($p < 0.05$).
- Because of its role in the conversion of testosterone to estradiol, abundance of aromatase was monitored. A reduction ($p < 0.05$) was observed in DD-treated snails (consistent with the drop in fecundity and estradiol levels) and a comparable level to control in glyphosate-treated snails (consistent with their high fecundity and estradiol levels).

Overall:
Results corresponding to both glyphosate and diquat dibromide are reported in the summary.
The study supports that Roundup and its constituents (glyphosate and DD) are endocrine disruptors.
RMS notes that the concentration tested (3.5 mg/L) is higher than the concentrations expected in real conditions of use. The report also states that a "low" level of spontaneous mortality occurred. It is not known to what extend this letality has affected the results (no biological data presented for mortality).
No MTC could be determined (no mortality data, only one tested concentration).
No analytical verification was conducted.
The results from this study (for glyphosate) are relevant for the assessment of potential for endocrine disruption.
The study is relevant but unreliable (as it cannot be excluded that effects are due to systemic toxicity).

Data point:	CA 9
Report author	Smith C. M. *et al.*
Report year	2019
Report title	Developmental and epigenetic effects of Roundup and glyphosate exposure on Japanese medaka (*Oryzias latipes*).
Document No	Aquatic toxicology (2019), Vol. 210, pp. 215-226
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities

Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

Full summary of the study according to OECD format

The study examined developmental teratogenic effects and adult-onset reproductive effects of exposure to environmentally relevant concentrations of glyphosate and Roundup in Japanese medaka fish (*Oryzias latipes*). Hd-rR strain medaka embryos were exposed to 0.5 mg/L glyphosate, 0.5 mg/L and 5 mg/L Roundup (glyphosate acid equivalent) for the first 15 days of their embryonic life and then allowed to sexually mature without further exposure. Whole body tissue samples were collected at 15 days post fertilization (dpf) and brain and gonad samples were collected in mature adults. Hatching success and phenotypic abnormalities were recorded up until 15 dpf. Roundup (0.5 mg/L) and glyphosate decreased cumulative hatching success, while glyphosate exposure increased developmental abnormalities in medaka fry. Expression of the maintenance DNA methyltransferase gene Dnmt1 decreased, whereas expression of methylcytosine dioxygenase genes (Tet1, Tet2 and Tet3) increased in fry at 15 dpf suggesting that epigenetic alterations increased global DNA demethylation in the developing fry. Fecundity and fertilization efficiency were not altered due to exposure. Among the reproduction-related genes in the brain, kisspeptin receptor (Gpr54-1) expression was significantly reduced in females exposed to 0.5 mg/L and 5 mg/L Roundup, and Gpr54-2 was reduced in the 0.5 mg/L Roundup treatment group. No change in expression of these genes was observed in the male brain. In the testes, expression of Fshr and Arα was significantly reduced in medaka exposed to 0.5 mg/L Roundup and glyphosate, while the expression of Dmrt1 and Dnmt1 was reduced in medaka exposed to 0.5 mg/L glyphosate. No change in expression of these genes was observed in the ovaries. The present study demonstrates that Roundup and its active ingredient glyphosate can induce developmental, reproductive, and epigenetic effects in fish; suggesting that ecological species, mainly fish, could be at risk for endocrine disruption in glyphosate and Roundup-contaminated water bodies.

Materials and methods

Fish maintenance
Hd-rR strain of the wild-type medaka fish (*Oryzias latipes*) were cultured in the Department of Biology at The University of North Carolina at Greensboro from an inbred line. Fish were allowed to mature in 38 liter tanks with constant aeration and water flow of approximately 76 liters/day. Water temperature was maintained at 25 ± 1 °C, while fish were kept under a light: dark cycle of 14 h: 10 h and fed TetraMin Tropical Flakes twice daily. Additional culture conditions include: pH: 6.8–7.2, total alkalinity: 0–40 ppm, nitrate: 0–20 ppm, nitrite: 0 ppm and ammonia: 0–0.25 ppm.

Chemical exposure and sampling
Upon maturation to adulthood (approximately 100-120 days), medaka were allowed to naturally spawn and eggs were gently collected by hand from mature females and placed into deionized water (DI) containing 0.05% methylene blue. Methylene blue treatment is necessary, as medaka embryos develop fungal infections if not treated. Collected eggs were then sorted and fertilized eggs were placed into non-treated, sterile, 48 well plates and exposed to DI water with 0.05% methylene blue for approximately 8 hours. At approximately 8 hours post fertilization (hpf) the water in each well plate was changed to the appropriate exposure solution. Exposure lasted from 8 hpf until 15 days post fertilization (dpf), with daily changes of 50% of the exposure media. Upon hatching, fry were transferred to petri dishes containing exposure media without methylene blue. At 15 dpf the exposure media in the petri dishes was changed to control media, with medaka later being allowed to reach sexual maturity in 38 liter tanks. For each treatment, given all replicates, number of fish (male and female combined) was maintained between 20 and 37, utilizing at least 3 tanks per treatment.

Nominal exposure concentrations included: control (DI water), 0.5 mg/L glyphosate and two concentrations of Roundup (glyphosate acid equivalent): 0.5 mg/L and 5 mg/L. The treatments have been abbreviated hereafter as Low Roundup, High Roundup, and glyphosate for 0.5 mg/L Roundup, 5.0 mg/L Roundup, and 0.5 mg/L glyphosate, respectively. Glyphosate (certified reference material) was acquired from Sigma-Aldrich (catalog number: 89432), while Roundup® Ready to Use (Monsanto) (hereinafter Roundup) was purchased from a local garden center. The exposure concentrations of 0.5 mg/L for the glyphosate treatment group and 0.5 mg/L Roundup (glyphosate acid equivalent), representing the Low Roundup treatment group, were chosen to represent an environmentally relevant concentration, given that the current maximum contaminant level (MCL) set by the U.S. Environmental Protection Agency (EPA) for glyphosate in drinking water in the United States is 0.7 mg/L (Battaglin et al., 2002; Monsanto, 2014). The higher exposure concentration of 5 mg/L Roundup was chosen to represent an upper bound given direct application or an occasional contamination event, as concentrations of Roundup (glyphosate acid equivalent) have been reported to range from 1.7 to 5.2 mg/L in areas following direct application or an accidental spill (Annett et al., 2014; Harayashiki et al., 2013).
Medaka samples were collected at 15 and 100 dpf. Fry samples collected at 15 dpf were euthanized using MS-220 (250 mg/L) before being flash frozen in liquid nitrogen. Upon reaching sexual maturity the remaining medaka fish were allowed to spawn naturally and eggs were collected from females (n = 10–11) for a period of seven consecutive days, in order to measure fecundity and fertilization efficiency. After egg collection ceased, the mature medaka were humanely sacrificed using MS-220 (250 mg/L) and the brain and gonads of adult male (n = 8–16) and adult female medaka (n = 9–12) were subsequently dissected and flash frozen in liquid nitrogen. All samples were stored at −80 °C until molecular analysis was performed.

Phenotype characterization
Embryos were examined daily until 15 dpf under a stereomicroscope and developmental abnormalities were recorded and photographed. Microscopic observations were performed using a Nikon SMZ1000 stereomicroscope equipped with a PlanApo objective; while pictures were captured using a Moticam 2300- 3 megapixel CMOS camera running under the Images Plus capture package. Pictures were later analyzed for changes in phenotype compared to control. Developmental abnormalities were measured by counting newly hatched fry that had at least one of the following abnormalitites: spinal curvature, enlarged yolk sac, uninflated swim bladder and greying of the yolk sac; while poor development was counted as individuals with at least three out of four developmental abnormalities.

Fecundity and fertilization efficiency
Upon maturation, remaining medaka in each replicate were placed into mating pairs at a ratio of 3 females: 2 males, unless death by treatment was high enough to not enable this ratio. Fish were allowed to naturally spawn and egg clutches were collected by hand from mating females approximately 1 hour after the lights turned on. Eggs were examined under the stereoscope and the mean number of eggs per female per day (fecundity) was calculated, along with whether the eggs were fertilized or not (fertilization efficiency), for a period of seven consecutive days. Statistical significance was determined using a two-tailed *T*-test against control with standard error.

RT-qPCR
Whole body tissue samples from 15 dpf fry, and isolated brain and gonad samples from both male and female adult medaka, were homogenized and DNA/RNA was extracted using the ZymoResearch Z-R Duet MiniPrep Kit protocol using DNAse I and proteinase K treatment. The purity and integrity of RNA for real time qPCR was measured using Thermo Scientific Nano Drop 2000/2000c Spectrophotometer. RNA was then reverse transcribed into cDNA using Applied Biosystems High Capacity cDNA Synthesis kits standard protocol. To assess gene expression, Power-Up SYBR Green Master reagents were used alongside primers that were specifically designed for the medaka genes of interest presented in the study (Table S1). mRNA levels were quantified using real-time qPCR (△△CT method) and gene expression data is presented as fold change against control.

Statistical analysis
Statistical significance was determined using two-tailed T-test against control. Differences in gene expression among exposure groups were determined by one-way ANOVA followed by a Tukey's post-hoc test. All data is expressed as the mean ± standard error of the mean (SEM).

Results

Developmental abnormalities
On the day of hatching, several developmental abnormalities were observed including: spinal curvature, enlarged yolk sac, uninflated swim bladder and greying of the yolk sac. One common phenotypic abnormality observed on the day of hatching was spinal curvature. The average rate of spinal curvature for the control, Low Roundup, High Roundup and glyphosate groups was 1.7%, 27.9%, 1.8% and 25%, respectively. Additionally, a fry was classified as "poor development" if it possessed three out of four developmental abnormalities. The average rate of fry qualifying as poor development for the control, Low Roundup, High Roundup and glyphosate treatment groups was 4.3%, 18.2%, 10.10%,and 33.2%, respectively. Overall, developmental defects were significantly increased in medaka exposed to glyphosate (*$p < 0.05$). No significant difference in mortality was observed from the day of hatch until maturation.

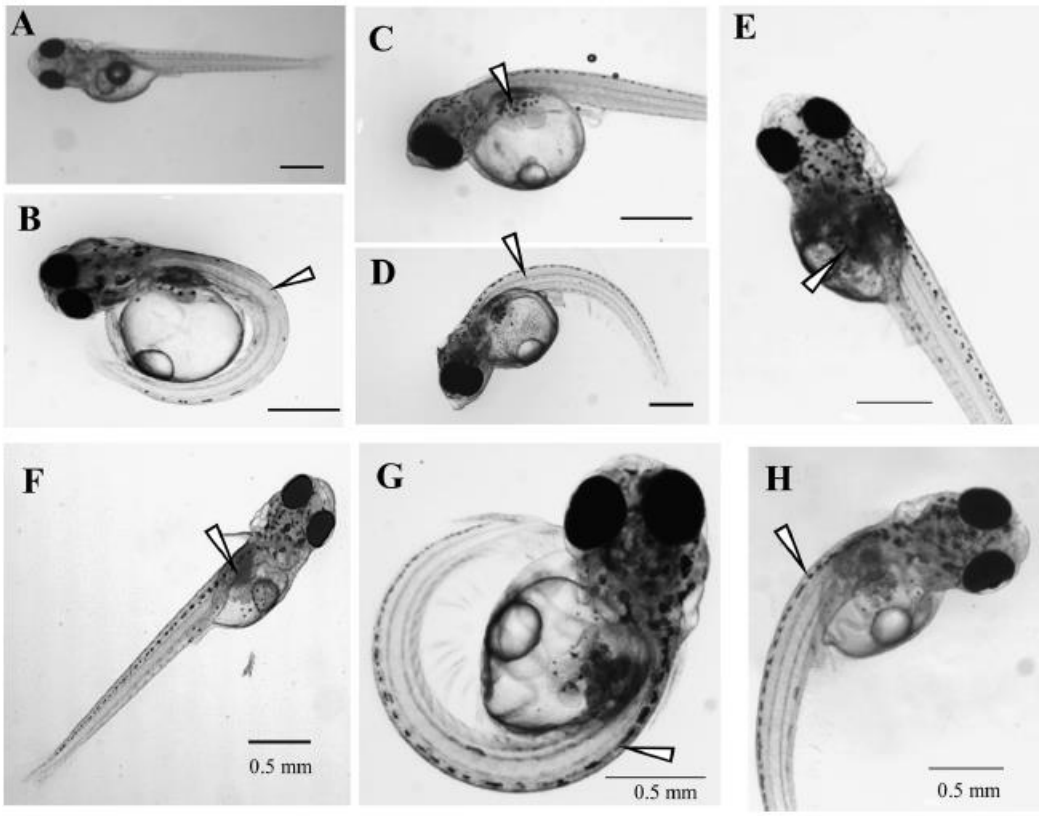


Fig. 1. Phenotypic abnormalities in medaka fry chronically exposed to glyphosate and Roundup formulations from the day of fertilization to 15 dpf. A. Control: normal embryo, B. 0.5 mg/L Roundup: embryo with spinal curvature that persisted throughout life, C–E. 0.5 mg/L Glyphosate: fry with uninflated swim bladder (C), fry with spinal curvature (D), fry with darkened yolk sac (E). F–G. 5 mg/L Roundup: embryo with uninflated swim bladder (F), spinal curvature (G), and mild spine curvature (H). Arrows indicate developmental abnormalities.

Hatching success
Cumulative hatching success (defined as hatching 7–12 dpf) was calculated for each treatment group. The average rate of hatching for the control, Low Roundup, High Roundup and glyphosate groups was 58.7%, 44.5%, 54.9% and 35.2% respectively. Compared to control, cumulative hatching success was significantly decreased(*$p < 0.05$) in medaka exposed to Low Roundup and glyphosate. Cumulative hatching success was then broken down into normal hatching(hatching between 7–10 dpf) and delayed

hatching (hatching between11–12 dpf). Hatching stages were chosen according to Iwamatsu (2004), which details the developmental stages of medaka. Embryos in the Low Roundup group showed a significant decrease in normal hatching rate and increase in delayed hatching rate(*$p < 0.05$)

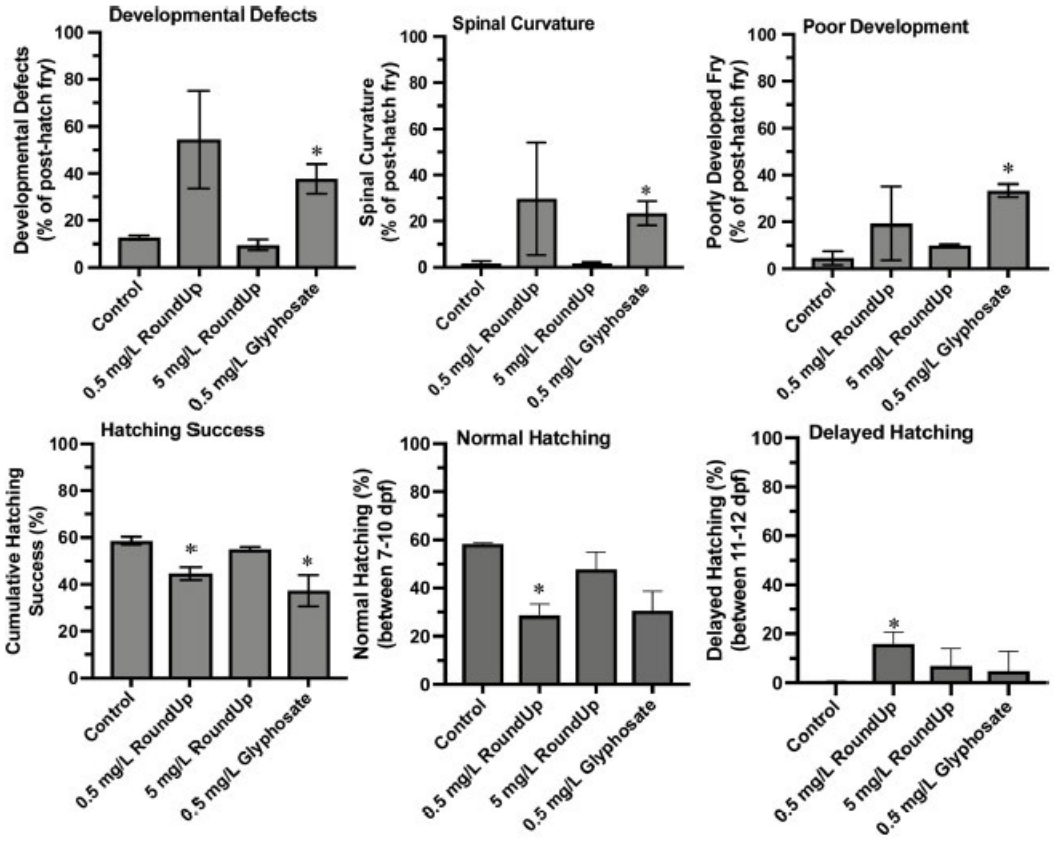


Fig. 2. Developmental abnormalities (upper panel) and hatching success (lower panel) in medaka chronically exposed for 15 days to Roundup and Glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (* $p < 0.05$).

Expression of epigenetic genes in 15 dpf fry

mRNA levels of Dnmt1, Dnmt3aa, Tet1, Tet2 and Tet3 were measured in fry at 15 dpf. mRNA levels of Dnmt1 were significantly decreased in the Low Roundup (**$p < 0.01$) and High Roundup (*$p < 0.05$) groups, while mRNA levels were at the significance level of $p < 0.51$ in the glyphosate group. mRNA levels for Tet1 and Tet3 were significantly increased in all exposure groups, while Tet2 was significantly increased in both the Low and High Roundup groups when compared to control. Comparing between treatments, Dnmt3aa, Tet2, and Tet3 mRNA levels were more significant when comparing between the glyphosate and High Roundup groups than when comparing between the Low Roundup and High Roundup groups (†$p < 0.05$;‡$p < 0.01$,†‡$p < 0.001$). Dnmt1 and Dnmt3aa mRNA levels were decreased in the glyphosate group, with marginal significance, compared to the control group ($^{\nabla}p < 0.05{\sim}0.1$).

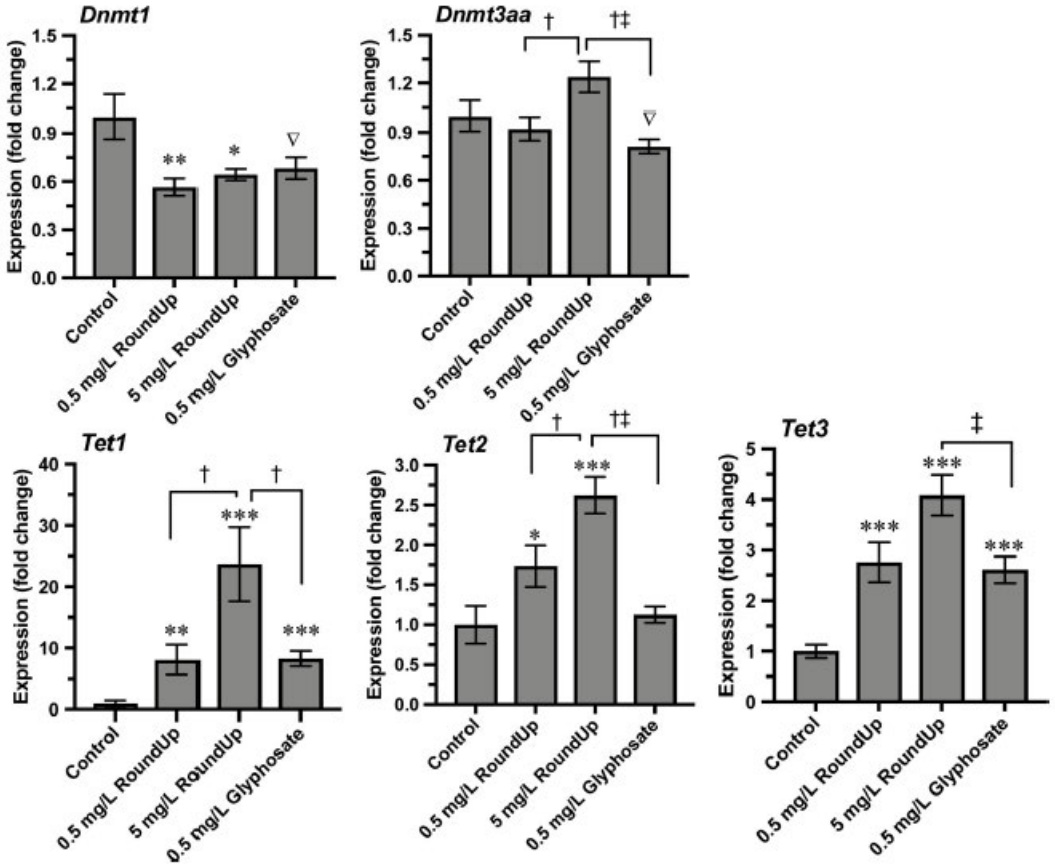


Fig. 3. Expression of epigenetic genes [DNA methyltransferase (Dnmt) and ten-eleven translocation (Tet)] in 15-dpf medaka fry chronically exposed to Roundup and glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, ∇ $p < 0.05$~0.1). Significant differences when compared among treatments are presented by symbols (†$p < 0.05$; ‡ $p < 0.01$; †‡ $p < 0.001$).

Fecundity and fertilization efficiency

After collection of samples at 15 dpf the remaining fry were raised to adulthood, without further exposure, to collect data on reproductive parameters. Fecundity and fertilization efficiency were determined in mature medaka. Fecundity, represented as the average number of eggs/female/day (mean ± SEM) for the control, Low Roundup, High Roundup and glyphosate groups was 5.5 ± 1.7, 6.8 ± 1.1, 6.3 ± 2.2 and 4.0 ± 1.3 respectively. Fertilization efficiency, re-presented as the average percent of fertilized eggs/replicate/day (mean ± SEM) was 63.4 ± 19.3, 58.6 ± 25.3, 47.5 ± 16.6 and 40.6 ± 20.5 for the control, Low Roundup, High Roundup and glyphosate groups respectively. Both fecundity and fertilization efficiency were not altered due to developmental exposure to either Roundup or glyphosate.

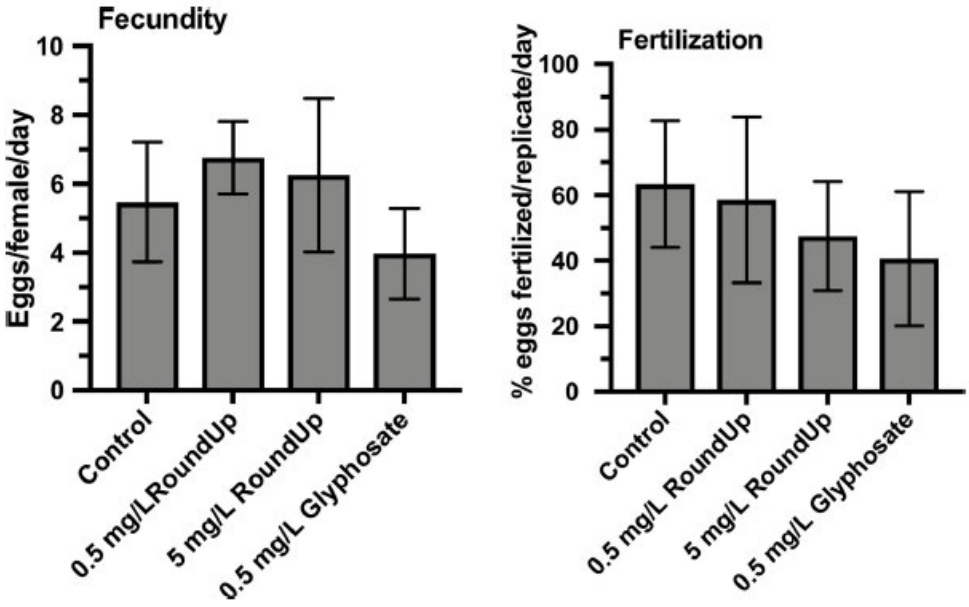


Fig. 4. Mean number of eggs laid per female per day and fertilization efficiency in adult medaka induced by 15-day chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM.

Expression of reproductive genes in the brain

In adult female brain samples the mRNA levels of Kiss1 and Kiss2 did not show any significant changes, while Gpr54-1was significantly downregulated in both the Low and High Roundup groups and Gpr54-2 mRNA levels were significantly downregulated in the Low Roundup group. Comparing between treatments, Gpr54-2 mRNA levels were equally as significant in the High Roundup as the glyphosate group, when compared to Low Roundup (‡ $p < 0.01$). In contrast, mRNA levels for Kiss1, Kiss2 and their receptors (Gpr54-1 and Gpr54-2) were not altered in any treatment group in adult male brain samples.

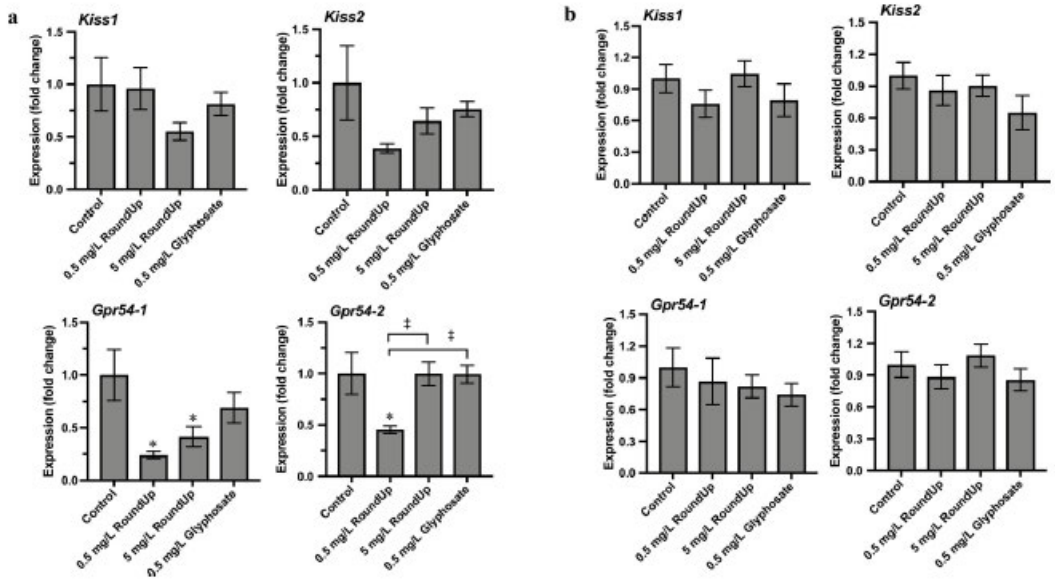


Fig. 5. (a) Expression of kisspeptin and kisspeptin receptor genes in the brain of adult female medaka chronically exposed during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (*$p < 0.05$) and when compared between treatments (‡ $p < 0.01$). Abbreviations: Kiss- Kisspeptin, Gpr54- Kisspeptin receptor. (b) Expression of kisspeptin and kisspeptin receptor genes in the brain of adult male medaka chronically exposed during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Abbreviations: Kiss- Kisspeptin, Gpr54- Kisspeptin receptor.

Expression of genes involved in sex differentiation and gonad development

The mRNA levels for Dmrt1 and Foxl2 were measured as an indicator of perturbation in sex differentiation. Dmrt1 mRNA levels were significantly decreased in the testes of the glyphosate group, while no change in Foxl2 mRNA levels were observed in the ovaries.

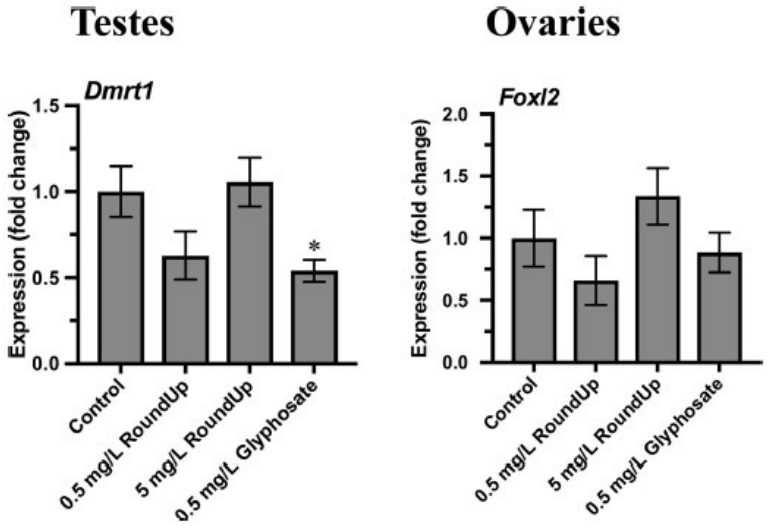


Fig. 6. Expression of genes involved in sex differentiation in response to chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (* $p < 0.05$). Abbreviations: *Dmrt1*- Doublesex and mab-3 related transcription factor 1 (involved in testes differentiation), *Foxl2*- Forkhead transcription factor L2 (involved in differentiation of ovaries).

Reproductive genes in the gonads

The mRNA levels for Star, Fshr, Lhr, Erα, and Cyp19a1a were determined in ovary samples at adulthood. No significant changes in mRNA levels were observed in the ovaries. In the testes, mRNA levels for Fshr, Lhr, Erα, and Arα were measured and mRNA levels for Fshr were significantly decreased in the testes of both the Low Roundup (**$p < 0.01$) and glyphosate group (*$p < 0.05$), compared to the control. Between treatments, the mRNA levels of Fshr in the male testes were more significant in the Low Roundup group than in the glyphosate group when compared to High Roundup (†$p < 0.05$;‡$p < 0.01$). Similar to the trend for Fshr, the mRNA levels for Arα were significantly decreased in the testes of both the Low Roundup and glyphosate group when compared to the control (*$p < 0.05$). No change in expression of Lhr or Erα was observed between the control and treatment groups in the male testes.

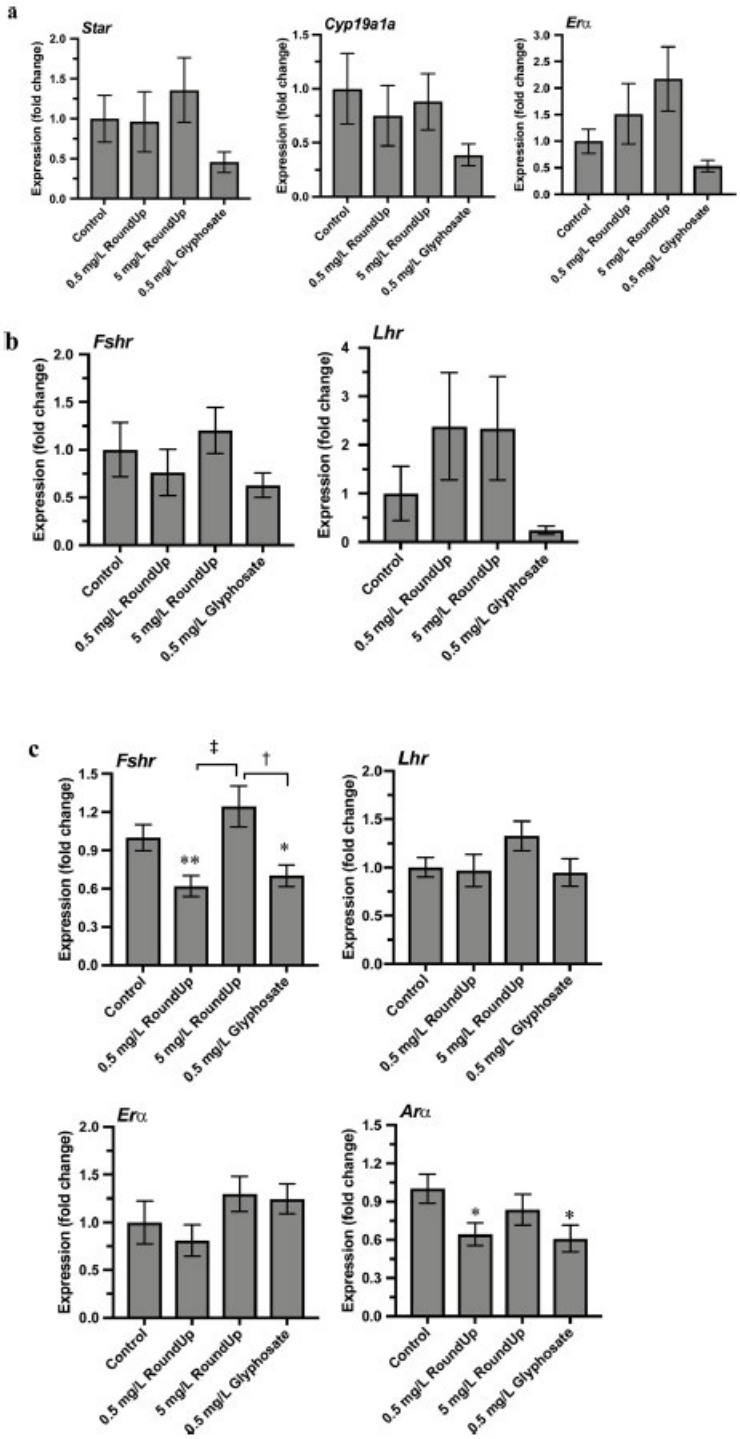


Fig. 7. (a) Expression of genes involved in female reproduction in the ovaries of adult female medaka in response to chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Abbreviations: Star-Steroidogenic acute regulatory protein, Cyp19a1a- Cytochrome P450 member 19 gene a1a subtype (Ovarian aromatase), Erα - Estrogen receptor alpha. (b) Expression of gonadotropin hormone receptor genes in the ovaries of adult female medaka in response to chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Abbreviations: Fshr- Follicle stimulating hormone receptor, Lhr- Luteinizing hormone receptor. (c) Expression of steroid hormone and gonadotropin hormone receptor genes in adult medaka testes in response to chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (*$p < 0.05$; ** $p < 0.01$) and when compared between treatments (†$p < 0.05$; ‡ $p < 0.01$). Abbreviations: Fshr- Follicle stimulating hormone receptor, Lhr- Luteinizing hormone receptor, Erα- Estrogen receptor alpha, Ara-Androgen receptor alpha.

Epigenetic gene expression

Alteration in mRNA levels for only Dnmt1 were used as a measure of global epigenetic changes in adult gonads. The mRNA levels for Dnmt1were decreased in the testes of the glyphosate group (**p < 0.01) compared to the control, whereas no significant change was observed in the ovaries.

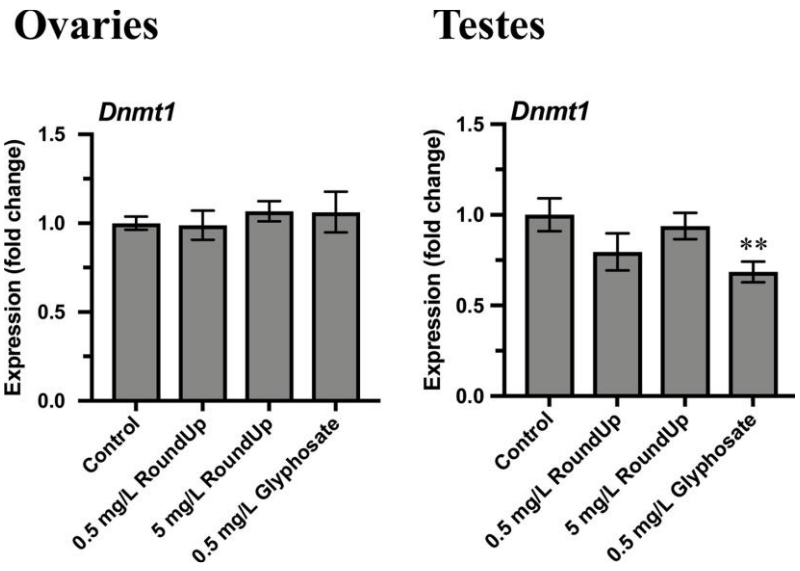


Fig. 8. Expression of epigenetic genes in adult medaka gonads in response to chronic exposure during early life to Roundup and glyphosate. Results are presented as mean ± SEM. Asterisks indicate statistical significance when compared to control (** p < 0.01). Abbreviations: Dnmt1- DNA methyltransferase 1 (the DNA methyltransferase enzyme that maintains DNA methylation on CpG site of the genome).

Conclusion

Roundup (0.5 mg/L) and glyphosate decreased cumulative hatching success, while glyphosate exposure increased developmental abnormalities in medaka fry. Expression of the maintenance DNA methyltransferase gene Dnmt1 decreased, whereas expression of methylcytosine dioxygenase genes (Tet1, Tet2 and Tet3) increased in fry at 15 dpf suggesting that epigenetic alterations increased global DNA demethylation in the developing fry. Fecundity and fertilization efficiency were not altered due to exposure. Among the reproduction-related genes in the brain, kisspeptin receptor (Gpr54-1) expression was significantly reduced in females exposed to 0.5 mg/L and 5 mg/L Roundup, and Gpr54-2 was reduced in the 0.5 mg/L Roundup treatment group. No change in expression of these genes was observed in the male brain. In the testes, expression of Fshr and Arα was significantly reduced in medaka exposed to 0.5 mg/L Roundup and glyphosate, while the expression of Dmrt1 and Dnmt1 was reduced in medaka exposed to 0.5 mg/L glyphosate. No change in expression of these genes was observed in the ovaries. The present study demonstrates that Roundup and its active ingredient glyphosate can induce developmental, reproductive, and epigenetic effects in fish; suggesting that ecological species, mainly fish, could be at risk for endocrine disruption in glyphosate and Roundup-contaminated water bodies.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: No specific endpoints are presented that could be applied to an EU level ecotoxicology risk assessment for Annex I renewal. Enzyme, cellular and molecular level endpoints are not relevant to EU level ecotoxicology risk assessment.
In this study only single application rates for glyphosate active substance were considered. The data on the formulation Roundup Ready are not relevant for the glyphosate EU renewal as it not the representative formulation MON 52276 for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The study examined developmental teratogenic effects and adult-onset reproductive effects of exposure to environmentally relevant concentrations of glyphosate and Roundup in Japanese medaka fish (Oryzias latipes). Hd-rR strain medaka embryos were exposed to 0.5 mg/L glyphosate, 0.5 mg/L and 5 mg/L Roundup (glyphosate acid equivalent) for the first 15 days of their embryonic life and then allowed to sexually mature without further exposure. Whole body tissue samples were collected at 15 days post fertilization (dpf) and brain and gonad samples were collected in mature adults. Hatching success and phenotypic abnormalities were recorded up until 15 dpf.

- Roundup (0.5 mg/L) and glyphosate decreased cumulative hatching success, while glyphosate exposure increased developmental abnormalities in medaka fry.
- Expression of the maintenance DNA methyltransferase gene Dnmt1 decreased, whereas expression of methylcytosine dioxygenase genes (Tet1, Tet2 and Tet3) increased in fry at 15 dpf suggesting that epigenetic alterations increased global DNA demethylation in the developing fry.
- Fecundity and fertilization efficiency were not altered due to exposure.
- Among the reproduction-related genes in the brain, kisspeptin receptor (Gpr54-1) expression was significantly reduced in females exposed to 0.5 mg/L and 5 mg/L Roundup, and Gpr54-2 was reduced in the 0.5 mg/L Roundup treatment group. No change in expression of these genes was observed in the male brain.
- In the testes, expression of Fshr and Arα was significantly reduced in medaka exposed to 0.5 mg/L Roundup and glyphosate, while the expression of Dmrt1 and Dnmt1 was reduced in medaka exposed to 0.5 mg/L glyphosate. No change in expression of these genes was observed in the ovaries.

RMS does not consider the results for Roundup relevant (surfactants may induce effects that cannot be discriminated). Results for glyphosate in isolation are relevant.

Overall for glyphosate, while there was no significant difference in reproductive mRNA expression levels in adult medaka ovary samples, a significant decrease in Fshr and Arα mRNA levels was observed in adult testes exposed to glyphosate treatment group, along with a significant decrease in Dmrt1 mRNA levels in the testes of medaka exposed to glyphosate. These results suggest that glyphosate affect the male reproductive system by modulating genes required for spermatogenesis.

RMS notes that hatching success was 58.7% in the control. This seems rather low even it has to be acknowledged that no standardized guideline/validity criteria was used to decide whether this rate is acceptable or not. The condition of the fish is questionable.
RMS notes that the present study showed an increased incidence of developmental abnormalities in medaka fry chronically exposed to 0.5 mg/L glyphosate, suggesting potential teratogenic effects. Systemic toxicity cannot be discarded.
Analytical verification was not made.
The decrease in Fshr and Arα mRNA levels observed in adult testes for glyphosate treatment group at 0.5 mg/L was not observed for Roundup at 5 mg/L. This is also the case for the decrease in Dmrt1 mRNA levels in the testes of medaka exposed to glyphosate (no decreased for Roundup at 5 mg/L).
The study authors hypothesized that glyphosate has the ability to display non-monotonic dose-responses taking the shape of a U-shaped curve (and that in the present study, the lack of response at 5 mg/L Roundup would actually correspond to an intermediate dose levels).
The number of tested concentrations are not sufficient to support this. RMS doubts the reliability of the conclusions of this study.

The results from this study (for glyphosate) are relevant for the assessment of potential for endocrine disruption. The study results are considered not reliable.

8. Bees

Data point:	CA 8.3.1.4, CP 10.3.1.4
Report author	Balbuena M. S. *et al*.
Report year	2015
Report title	Effects of sublethal doses of glyphosate on honeybee navigation.
Document Source	The Journal of experimental biology (2015), Vol. 218, No. 17, pp. 2799
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

In this study, it was tested whether exposure to three sub lethal concentrations of glyphosate (GLY) (2.5, 5 and 10 mg/L: corresponding to 0.125, 0.250 and 0.500 µg per animal) affects the homeward flight path of honeybees in an open field. An experiment was performed in which forager honeybees were trained to an artificial feeder, and then captured, fed with sugar solution containing traces of GLY and released from a novel site either once or twice. Their homeward trajectories were tracked using harmonic radar technology. Honeybees that had been fed with solution containing 10 mg/L GLY spent more time performing homeward flights than control bees or bees treated with lower concentrations. They also performed more indirect homing flights. Moreover, the proportion of direct homeward flights performed after a second release from the same site increased in control bees but not in treated bees.

Materials and methods

Animals and study site
Bees used from a colony of approximately 30,000 bees (*Apis mellifera* Linnaeus 1758). The experiment was conducted from August to September of 2013 in an open field (N 50°48′53.01″, E 8°52′21.36″) located close to the village of Großseelheim (Hessen), Germany.

Experimental procedure
A group of forager bees was trained to collect unscented 0.5 mol/L sucrose solution from an artificial feeder located 400 m north of the hive and fitted with colored number tags on the thorax for individual identification. At 15 min intervals, numbered bees were captured individually at the feeder before they

began to ingest the sucrose solution offered and were immediately confined in plastic tubes, and transported to the release site (RS) located 460 m east of the feeder location. The RS was located within the area explored during orientation flights, but otherwise it was novel for the trained bees. Each plastic tube contained a small feeder providing 50 µl of unscented 2 mol/L sucrose solution, either with or without glyphosate. The tube was kept in a dark box for 1 hour (incubation), allowing bees to ingest all the solution offered. Three different concentrations of GLY were used (diluted in 2 mol/L sucrose solution): 2.5 mg/L, 5 mg/L and 10 mg/L. Control bees were handled in the same way but were fed the solution without herbicide (0 mg/L).
After incubation, a radar transponder was glued to the number tag fixed on the thorax of each bee and the homeward flight trajectory (from the RS to the hive) was tracked with harmonic radar. Bees were released at 15 min intervals to ensure the same incubation time for all the individuals. One experimenter at the radar station passed on information about the flight trajectories of the released bees by walkie-talkie. Once the bee arrived at the hive, it was caught, the transponder was removed, and then the honeybee was allowed to enter the hive. Whenever possible, these bees were captured at the feeder and released from the RS once more ('second release') in order to test whether learning during homing flights was compromised. The total number of bees tested was 108 for the first release, and 44 for the second. The number of flight trajectories obtained was 79 for first release and 37 for the second.

The following variables were measured: capture time, release time, arrival time at the hive and the flight trajectory recorded with the harmonic radar. If a bee was observed on the radar but then disappeared from the radar range and was not seen arriving at the hive, it was classified as a non-arriving bee.

Herbicide
A stock solution of glyphosate (Glyphosate PESTANAL, Sigma-Aldrich, Steinheim, Germany) at a concentration of 100 mg acid equivalent/L was prepared with distilled water and kept refrigerated. New stock solution was prepared every 7 days. The stock solution was diluted in sucrose solution 2 mol/L to obtain the different GLY concentrations used in the experimental procedure. The concentrations of herbicide used were: 0 mg (control), 2.5 mg, 5 mg and 10 mg of glyphosate per liter of sucrose solution. Each bee ingested 50 µl of 2 mol/L sucrose solution with or without GLY, so the concentrations used were equivalent to the following doses: 0 ng, 125 ng, 250 ng and 500 ng of glyphosate per bee.

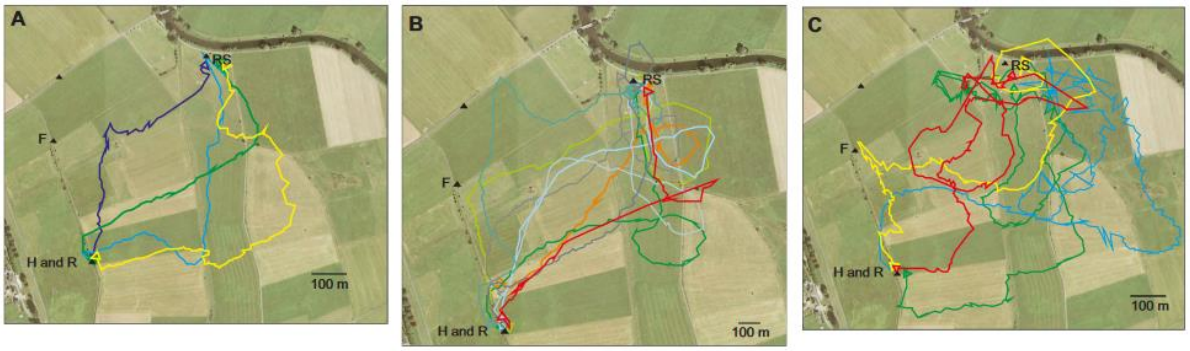
Harmonic radar tracking
Tracking bees with a harmonic radar system is described in Riley et al. (1996, 2005), Menzel et al. (2011) and Scheiner et al. (2013). Here, the authors used a system with a sending unit which consisted of a 9.4 GHz radar transceiver combined with a parabolic antenna of ~44 dBi that provided a signal from the transponder on the bee thorax every 3 s. The transponder consisted of a dipole antenna with a Low Barrier Schottky Diode HSCH-5340 of centered inductivity. The second harmonic component of the signal (18.8 GHz) was the target for the radar. The receiving unit consisted of an 18.8 GHz parabolic antenna with a low-noise preamplifier directly coupled to a mixer (18.8 GHz oscillator) and a downstream amplifier with a 90 MHz ZF-Filter. The transponder was made of a silver wire with a diameter of 0.3 mm, a length of 11 mm, a weight of 10.5 mg and a loop inductance of 1.3 nH. The range of the harmonic radar had a radius of 900 m.

Statistical analysis
A heterogeneity chi-square analysis was used to compare the proportion of bees performing direct or indirect flights from the release site back to the hive. A Kruskal-Wallis test was performed to compare the time bees spent between the RS and the hive, according to the treatments (control bees and bees exposed to GLY: 2.5 mg/L, 5 mg/L and 10 mg/L). To compare the proportion of bees performing direct or indirect flights according to whether bees were released once or twice, we applied Fisher's exact test.

Results

In a catch-and-release experiment as performed here, the authors expect that bees captured at the feeder and then released from the release site (RS) are motivated to return to the hive. After ingesting food contaminated with glyphosate, it was expected that these treated bees would perform irregular homeward flights or at least take more time than untreated control bees to return to the hive. The results show that animals either start immediately with a straight flight from the release site (Fig. 1A, B) or they perform less regular flights (Fig. 1C). Some of the straight flights follow the vector the bees would have taken if they had not been relocated to the release site. These flights were either directed towards the hive and finished at the hive, or they were directed towards the feeder and then followed the trained route from the feeder to the hive (Fig. 1A). Some of these initially straight flights at the beginning of their homing behavior were followed by a single loop before the bees return to the hive (Fig. 1B). Therefore, it was distinguished between two major flight categories: direct flights (straight flight with or without one loop, Fig. 1A, B) and indirect flights (flights with loops Fig. 1C).

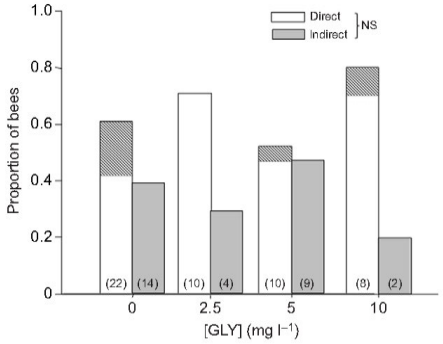
Figure 1. Examples of homeward flights made by honeybees during the first release after treatment. Flight paths were categorized as direct (A), single-loop (B) or indirect (C). Colors: light blue and red for control bees, blue and orange for bees treated with 2.5 mg/L glyphosate (GLY), yellow and lilac for bees treated with 5 mg/L GLY, and green and gray for bees treated with 10 mg/L GLY. H, hive; R, radar; F, feeder; RS, release site.



First release

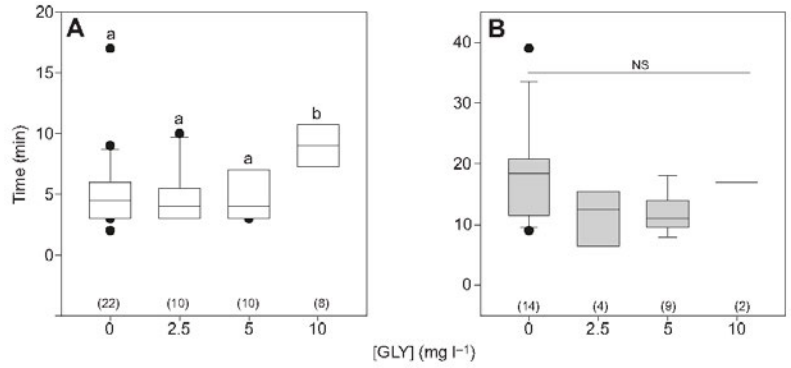
Fig. 2 shows the proportion of bees performing different homeward flights after being relocated from the feeder to the RS and released from the RS for the first time. As already mentioned, these homeward paths involve: (1) straight and rapid flights directly to the hive, with or without a single loop before returning to the hive (‘direct flights’); or (2) irregular flights, in which bees changed direction frequently (‘indirect flights’). Both control and treated bees showed similar proportions of direct flights to the hive.

Figure 2. Proportion of bees performing direct and indirect homeward flights after the first release. Proportion of bees performing direct and indirect homeward flights were pooled according to the treatment; looped flights are indicated by hatched bars. 0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to different concentrations of GLY (corresponding to 0.125, 0.25 and 0.5 µg per animal). NS, no significant difference (P>0.05). Numbers inside bars indicate the number of bees assessed for each treatment.



However, statistical differences in the time spent performing direct flights between treatments were found (Fig. 3A). Specifically, bees that had ingested sucrose solution containing 10 mg/L GLY spent more time flying from the RS to the hive than control bees or bees that had ingested 2.5 or 5 mg/L GLY. No statistical difference in the flight time was found between control and treated bees performing indirect flights (Fig. 3B).

Figure 3. Timing of homeward flights after the first release. Flying times from the release site to the hive according to different treatments (0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to 2.5 mg/L, 5 mg/L and 10 mg/L GLY, respectively). (A) Direct and 'single-loop' flights. (B) Indirect flights. Boxes with different letters are significantly different at P<0.05. NS, no significant differences (P>0.05). Numbers in brackets indicate the number of bees assessed for each treatment.



It was observed that during some homeward flights a small number of bees passed through the feeder area. The proportion of bees that flew via the feeder was higher among control bees and bees that ingested sucrose solution with 2.5 mg/L GLY than among bees treated with 5 or 10 mg/L (Table 1). After flying close to the feeder, those bees followed the trained flight route to the hive.

Table 1. Data for control and GLY-treated bees released for the first time

GLY treatment	No. of bees released	No. arrived at hive	No. arrived at hive via feeder	No. not arrived
0 mg l^{-1}	46	36 (0.78)	6 (0.17)	10 (0.22)
2.5 mg l^{-1}	25	14 (0.56)	6 (0.42)	11 (0.44)
5 mg l^{-1}	22	19 (0.86)	2 (0.11)	3 (0.14)
10 mg l^{-1}	15	10 (0.67)	2 (0.2)	5 (0.33)

Numbers in parentheses indicate the proportion of bees for each treatment.

Second release

Bees learn to improve their homing flights during sequential releases from the same site (Menzel et al., 2005). Therefore, next it was asked whether this form of learning is compromised in bees that have been exposed to the herbicide. To test this, bees were captured at the feeder, relocated to the RS, and released for a second time: these bees were therefore exposed twice to the same amount of GLY (Table 2).

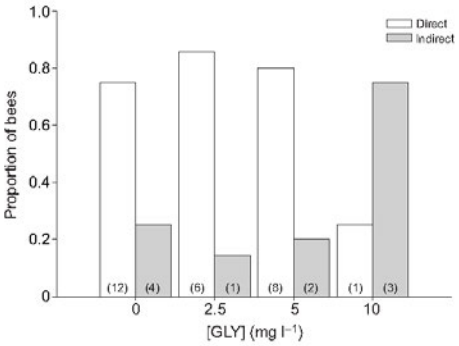
Table 2. Data for control and GLY-treated bees released for the second time

GLY treatment	No. of bees released	No. arrived at hive	No. not arrived
0 mg l^{-1}	19	16 (0.84)	3 (0.16)
2.5 mg l^{-1}	11	7 (0.64)	4 (0.36)
5 mg l^{-1}	10	10 (1)	0 (0)
10 mg l^{-1}	4	4 (1)	0 (0)

Numbers in parentheses indicate the proportion of bees for each treatment.

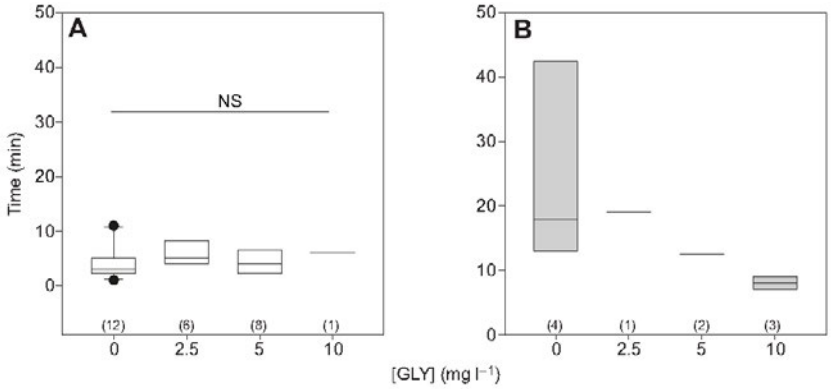
Control bees and bees that were exposed to 2.5 or 5 mg/L GLY showed a tendency to perform direct flights more frequently than indirect flights (Fig. 4). Conversely, bees that had ingested sucrose solution with 10 mg/L GLY showed the inverse tendency, with more bees performing indirect flights.

Figure 4. Proportion of bees performing direct and indirect homeward flights after the second release. Numbers in brackets indicate the number of bees assessed for each treatment.



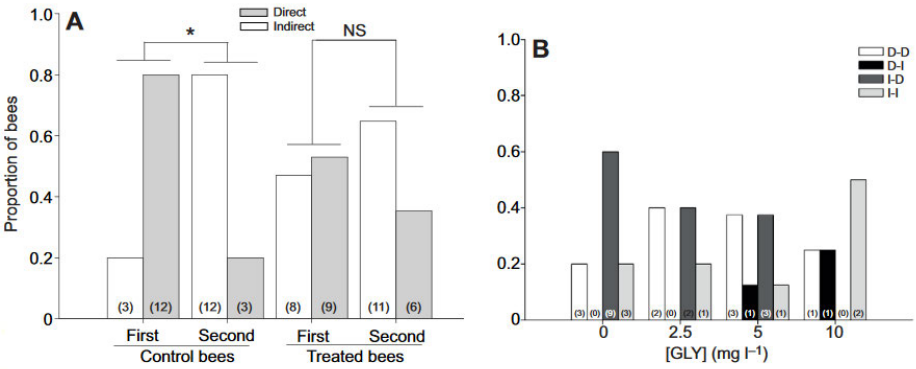
Nevertheless, no statistical differences for the time spent in direct flights were found between control and treated bees (Fig. 5A). It was not possible to perform a statistical analysis of data for indirect flights (Fig. 5B) because the sample size was too small.

Figure 5. Timing of homeward flights of bees after the second release. Flying times from the release site to the hive represented for the different treatments (0 mg/L: control bees; 2.5 mg/L, 5 mg/L and 10 mg/L: bees exposed to 2.5 mg/L, 5 mg/L and 10 mg/L GLY, respectively). (A) Direct flights. (B) Indirect flights. NS, no significant differences (P>0.05). Numbers in brackets indicate the number of bees assessed for each treatment.



When the proportion of control and treated bees that performed direct and indirect flights after the first and second release was compared, statistical differences between control bees released once or twice were found, but not between treated bees (Fig. 6A). Control bees modified their tendency to perform more indirect flights after the first release than after the second one, whereas the proportion of treated bees performing direct or indirect flights after one or two releases was similar. Furthermore, when studying the transitions (or lack thereof) from direct or indirect flights (or vice versa) performed after the first release to direct or indirect flights performed after the second release (direct-direct: D-D, direct-indirect: D-I, indirect-direct: I-D and indirect-indirect: I-I), a tendency for control bees was observed to perform more I-D transitions than treated bees. Interestingly, bees that had ingested the higher GLY concentration showed a tendency to perform more transitions to indirect flights (D-I, I-I) after the second release (Fig. 6B).

Figure 6. Proportion of transitions in performance after the first and second release according to treatment. (A) Control and treated bees were categorized according to direct (white bars) or indirect flights (gray bars) after the first release (fed GLY once) or the second release (fed GLY twice). (B) Flight transitions between the first and second release was considered per experimental bee (D: direct flight, I: indirect flight). The categories of both flights were: D-D (both flights were direct), I-I (both flights were indirect), D-I (the first flight was direct and the second indirect), I-D (the first flight was indirect and the second direct). *P<0.05; NS, no significant difference (P>0.05). Numbers in brackets indicate the number of bees assessed for each treatment.



Conclusion

Despite the lack of data on the actual level of GLY that forager honeybees are exposed to in the field, present results show that exposure to non-lethal concentrations of glyphosate causes sub-lethal effects, which modify the bees' foraging behavior. However, further studies are necessary to evaluate to what extent this chemical influences foraging behavior of honeybees in a natural environment and whether

prolonged exposure to this herbicide might contribute to worsen the health status of beehives. Since GM herbicide-tolerant crop fields are usually surrounded by native flora that is visited by honeybees, it would also be necessary to analyze traces of glyphosate present in collected and stored honey and pollen, as well as in larvae and adult bees from hives located in the surroundings of agricultural crops treated with GLY, before and after the herbicide application.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Due to the foraging nature of bees in the natural environment described in this study, the effects cannot be solely attributed to glyphosate active substance. However, the impact of bees from other substances foraging during the homing flight is considered negligible as they were exposed to the test substance for 1 hour prior to release. Furthermore, there is no clear indication of the dose of glyphosate that the bees were exposed to as there was no analytical verification conducted in the study. This is a new experimental design and does not provide relevant endpoints for the ecotoxicological regulatory risk assessment / glyphosate EU renewal. As there is no test guideline to which this study can be compared, and the data generated using this new experimental design and as there is no data requirement nor approach to evaluate the findings of such a study at the regulatory level, the findings of this study should be considered with a degree of caution. The reliability assessment highlights that elements of the study may be considered reliable, but there are no validity criteria against which this study can be assessed, nor data requirements relating to the achieved endpoints.

Assessment and conclusion by RMS:

In this study homeward trajectories of honeybees were tracked using harmonic radar technology. The researchers distinguished between two major flight categories: direct flights (straight flight with or without one loop) and indirect flights (more than one loop).
Honeybees that had been fed with solution containing 10 $mg.l^{-1}$ glyphosate spent more time performing homeward flights than control bees or bees treated with lower concentrations. They also performed more indirect homing flights. The proportion of direct homeward flights performed after a second release from the same site increased in control bees but not in treated bees. These results suggest that, in honeybees, exposure to glyphosate impairs the cognitive capacities needed to retrieve and integrate spatial information for a successful return to the hive. LOEL = 10 mg glyphosate/L in sucrose solution.
RMS notes the absence of clear conceptual link between flight time and the specific protection goals for bees (SPG). It is agreed that a longer duration of foraging trips may play a role in the colony/population health, but such link is not immediate in conceptual terms and not quantifiable. The results of this study are not directly relevant for risk assessment purpose but still relevant to address "other effects".
Reliability was not assessed in depth but results are considered unreliable. The study states that bees fed with solution containing 10 $mg.l^{-1}$ glyphosate spent more time performing homeward flights than control bees or bees treated with lower concentrations and also performed more indirect homing flights.
After first release
RMS notes that a significant proportion of control bees made indirect flight meaning that such indirect flight might not be considered necessarily as an adverse effect. Both control and treated bees showed similar proportions of direct flights to the hive (no statistical difference).
However statistical differences in the time spent performing direct flights between treatments were noted at 10mg/L. However this statement is based on only 8 bees.
No statistical difference in the flight time was found between control and treated bees performing indirect flights (only 2 bees at 10mg/L). RMS notes that (in this indirect flights category) flight time even seems to decrease in treated bees.

After second release
Control bees and bees that were exposed to 2.5 or 5 mg/L GLY showed a tendency to perform direct flights more frequently than indirect flights. Conversely, bees that had ingested sucrose solution with 10 mg/L GLY showed the inverse tendency, with more bees performing indirect flights. RMS notes that this statement is based on 4 bees (1 direct vs 3 indirect).
Overall the number of bees used in the experiments were very low and not sufficient to represent the natural variability of the parameters investigated.
Overall, the study is relevant and not reliable.

Data point:	CA 8.3.1
Report author	Vazquez D. E. et al.
Report year	2018
Report title	Glyphosate affects the larval development of honey bees depending on the susceptibility of colonies.
Document Source	PLoS One (2018), Vol. 13, No. 10, pp. E0205074
Guidelines followed in study	None
Deviations from current test guideline	Not applicable

GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The study aimed to assess the effects of glyphosate (GLY) on larval development under chronic exposure during *in vitro* rearing. Even though this procedure does not account for social compensatory mechanisms such as brood care by adult workers, it allows us to control the herbicide dose, homogenize nutrition and minimize environmental stress. The results showed that brood fed with food containing GLY traces (1.25±5.0 mg per litre of food) had a higher proportion of larvae with delayed moulting and reduced weight. The assessment also indicates a non-monotonic dose-response and variability in the effects among colonies. Differences in genetic diversity could explain the variation in susceptibility to GLY. Accordingly, the transcription of immune/detoxifying genes in the guts of larvae exposed to GLY was variably regulated among the colonies studied. Consequently, under laboratory conditions, the response of honey bees to GLY indicates that it is a stressor that affects larval development depending on individual and colony susceptibility.

Materials and methods

Study site and animals
Experiments were performed from January to March during the summer season of the southern hemisphere. Larvae were sampled from six disease-free colonies (A-C in 2014 and D-F in 2015) of western honey bees (*Apis mellifera ligustica* Spinola) and reared *in vitro* (see below). Colonies were purchased from a commercial queen producer in November of each year and housed in new Langstroth hives at the experimental apiary of the University of Buenos Aires, Buenos Aires, Argentina. The new six queens were not genetically related (different parents; i.e., inter-colony genetic diversity) and they were naturally inseminated by multiple mates during free flights in the field (i.e., intra-colony genetic diversity).

In vitro rearing
An empty frame into the source colony (A-F) was introduces and monitored for 8 hours until the queen had laid enough eggs. Three days later the brood frame was withdrawn and carried to a room suitable for grafting. Around 120 first stadium larvae (0±8 hour old post-hatching) was grafted from the brood frame to plastic cups and placed in Petri dishes. This amount of larvae represented around 10% of the cohort (eggs laid in one day by the queen) and up to 1% of the colony. To avoid variability in grafting effect the same researcher carried out this procedure. Larvae were reared inside an incubator with constant temperature and relative humidity (34.5 °C and 95%, respectively) for five days. In order to prevent bacterial or fungal contamination and subsequent infection, sterile conditions were maintained and dead ones were removed daily. To standardize larval food administration, 160 µl of food was provided spread in six aliquots of increasing volume to each larva during the six days of feeding period: 10 µl during grafting, 10 µl at 24 h, 20 µl at 48 h, 30 µl at 72 h, 40 µl at 96 h and 50 µl at 120 h. A previously established diet was used: 6% D-glucose, 6% D-fructose and 50% commercial royal jelly. One grafting session was performed for each source colony per treatment every 2±3 days in order to exclude seasonal differences.

Exposure to GLY

To evaluate the effect of exposure to GLY (analytical standard, purity of 99.2%) in larva reared *in vitro*, the worst-case exposure scenario was assumed. The concentration of GLY in brood food during field exposure is not known but GLY is actually ingested by larvae. There is no biochemical procedure yet to measure accurately traces of GLY in royal jelly. Consequently, a chronic exposure to GLY was chosen considering environmental concentrations measured in natural and agricultural landscapes when recommended or excessive applications of the herbicide were used. Four treatments were defined: control group (food without herbicide), 1.25, 2.5 and 5 mg a.e. of GLY per litre of food. To prepare the food mixture for each concentration, we diluted a stock solution of 100 mg a.e./L which was renewed once a week due to slight photodegradation of GLY. Finally, the pH of the food mixture of each treatment during the experiment was measured.

Larval development
Throughout the growth/feeding period, four moults allow a honey bee larva to increase in size, which determines five stadia. A moult normally occurs around every 24 hours up to the 4-day post-hatching (96 h). Each stadium can be identified daily by the head diameter of the larva and its morphology. When a larva has a smaller size or different characteristics from the stadium it is expected to be in, it was classified as delayed.

Survival
Larvae can be classified as dead when their colour changes to brownish and they develop oedema, remain immobile and/or do not react to the contact of a paintbrush. Note of their status was taken daily.

Food ingestion and growth
At the end of the feeding period (fifth stadium), larvae eat all offered food in both rearing contexts. Furthermore, inside the hive and prior to pupation, cells are sealed by nursing bees at around 120 hours after hatching. Hence, it was sampled and weighed 5-day old larvae with complete food intake (110 µl of food ingested) from three colonies (D-F) to compare growth among the different GLY concentrations (10 larvae per concentration and colony). An electronic balance (Mettler Toledo AG285, ±0.1 mg) was used and the diameter of the head with a stereomicroscope (Leica MZ8) was measured for a morphometric identification of the instar. As there are environmental, nutritional and social differences with in-hive rearing, larvae from the same cohort was sampled to compare nutritional baseline between contexts. For inhive rearing, in the three colonies ten 5-day old larvae from sealed cells were sampled prior to spinning (i.e. larvae remained at the bottom of the cell before changes its position).

Dissection of the gut
The expression profile of some genes in the gut was analyzed as a parameter to assess brood defences and health. For this, 5-day old larvae in the fifth stadium from three colonies were sampled (D-F, in both rearing contexts as above section) with complete food intake (same dosage of GLY and nutritional status. They were dissected under a stereomicroscope and pooled 10 guts for each rearing context and each GLY treatment per colony. Pooled guts were immediately frozen in liquid nitrogen and stored at - 80 °C until RNA extraction.

RNA extraction
Larval gut samples were homogenized using a pestle and mortar in the presence of liquid nitrogen. Following the manufacturer's instructions, polyadenylated RNA was extracted using the mRNA Isolation Kit (Roche Molecular Biochemicals). The concentration of mRNA was quantified using the Qubit fluorometer (Invitrogen).

RT-PCR procedure
Transcript accumulation of all genes through a semi-quantitative procedure with RT-PCR was estimated. cDNA was synthesized with 50 ng of mRNA per sample by means of the Revert AidTM M-Mul V Reverse transcriptase system (Fermentas International Inc.). PCRs were performed using GoTaq DNA polymerase (Promega). PCR specific primers for all analysed genes were designed for conserved nucleotide sequences. Annealing conditions for each primer pair were optimized empirically to

determine the linear range of amplification. *Actin* was used as an endogenous control to normalize the amount of starting template.

Gene expression analysis
RT-PCR products of the target genes were separated and the endogenous control in 1.5% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium) and visualized by the UVP Doc-It LS Image Acquisition Software. The intensity of the bands was measured and compared against a standard molecular marker loaded on the same gel (100-bp DNA ladder, Invitrogen). To analyse the response of each gene in exposure to glyphosate, their expression levels were relativized to their control baseline level.

Statistics
Data analysis and graphics were performed in R. Survival and developmental data were analyzed with Accelerated failure-time models (ATF). Weighing data were analyzed with generalized linear models (GLM). Because of head diameter reached non-normality errors with different distributions, a Kruskal-Wallis test was performed. Hierarchical cluster analysis was performed with multiscale bootstrap resampling p-values to classify the genetic responsiveness for clustering in each edge. The alpha level was set at 0.05 and p-value corrected for multiple *post hoc* comparisons with Bonferroni procedure.

Results

Changes in larval development of honey bees exposed to GLY
In order to detect changes in honey bee larval development associated with chronic exposure to GLY, the authors quantified survival and successful moulting during the larval feeding period (i.e. during the first 120 hours post-hatching. Due to the possibility of variation among larvae from different source colonies in response to the herbicide, the authors sampled brood from 6 colonies (A-F) to rear them under *in vitro* conditions. The mean age of death of all larvae in the control group was 92.9 ± 34 h, around the last moult before the fifth stadium. For larvae exposed to GLY, the mean age of death was similar, 92.2-106.6 h. Nevertheless, the results show effects on survival proportion with a significant interaction between the source colony of each larva and the GLY concentration administered (ATF model: survival prop. ~ [GLY] + colony + [GLY] × colony, χ^2 (23) = 344.63, P < 0.001, N = 3062, *post-hoc* pairwise comparisons were performed with the log-rank test. Therefore, GLY affects larval survival with different patterns of dose-response among colonies (Fig 1). Colonies B, D and E did not show an adverse effect on survival, while the rest of the colonies showed a significantly lower survival after exposure to GLY (20-66% less of the baseline, Table 1). However, in colonies C and E, some GLY concentrations increased survival (22-39% more of the baseline, Table 1). The authors also found that the baseline of survival proportion was significantly different among colonies under *in vitro* rearing, when the authors compared the survival control curves (Table 1). The developmental process can have sublethal adverse effects such as delayed moulting. The mean age of delay for larvae of the control group was 65.8 ± 35 h. This is consistent with the switch of diet within the hive from worker jelly to bee bread. The mean age of delay for larvae treated with GLY was similar, 60.7-68.2 h. Again, the results show a significant interaction between the source colony of each larva and the GLY concentration administered (ATF model: prop. of successful moulting ~ [GLY] + colony + [GLY] × colony. χ^2 (23) = 409.2, P < 0.001, N = 3062, *post-hoc* pairwise comparisons were performed with the log-rank test). Therefore, GLY affects larval development with different patterns of dose-response among colonies (Fig 2). During *in vitro* rearing a number of unexposed larvae (22-46%) showed delay in the moulting process with variability among colonies (Table 1). Under GLY exposure all the colonies, except for B and E, showed an increase in the proportion of larvae with delayed moulting (52-184% more of the baseline, Table 1). Only one concentration in colony F showed a reduced delay in development (46% less of the baseline, Table 1). The same proportion of larvae showed a double effect (i.e., delayed moulting followed by death) regardless of GLY concentration (33% for the control group and 26-38% for GLY exposed groups). The effects on survival and development have shown a non-monotonic dose-response relationship with positive effects in certain cases. The lowest concentration of GLY induced early sublethal adverse effects in some colonies (C and D) after 72 h while the exposure concentrations

of 2.5 and 5.0 mg/L induced sublethal and lethal effects in several colonies (A, C, D and F). This pattern could be a consequence of a response threshold in the detoxifying process. Accordingly, at the end of the exposure period assessed (120 h), the larvae exposed to 1.25 mg/L of GLY reached doses of around 100 ng of GLY per larva. Meanwhile, larvae exposed to 2.5 and 5 mg/L of GLY reached that dose within 72 h of exposure.

Table 1. Variability among honey bee colonies in GLY effects.

Treatment	Effect (120 h)*	Year	2014			2015		
		Colony	A	B	C	D	E	F
Control	Baseline	Survival	0.86^{ab}	0.77^{bc}	0.61^{c}	0.95^{bc}	0.75^{bc}	0.89^{ab}
		Successful moulting	0.59^{a}	0.56^{a}	0.74^{ab}	0.78^{b}	0.61^{ab}	0.54^{a}
	Sample size		123	109	114	130	135	114
GLY exposure	Negative	LC (mg L^{-1}) †	5.0	NOEL	2.5	NOEL	NOEL	2.5
		Survival	0.29	-	0.38	-	-	0.71
		SLC (mg L^{-1}) †	2.5	NOEL	1.25–5.0	1.25–5.0	NOEL	2.5
		Successful moulting	0.35	-	0.00–0.48	0.21–0.52	-	0.30
	Positive	EC (mg L^{-1}) †	NOEL	NOEL	1.25, 5.0	NOEL	2.5	5.0
		Survival	-	-	0.85	-	0.92	-
		Successful moulting	-	-	-	-	-	0.75
	Range of sample size		119–156	119–123	119–144	126–137	124–139	126–140

*Cumulative proportion of larvae with developmental effects during the exposure period to GLY (1.25, 2.5 and 5.0 mg of GLY per litre of food). The endpoints are death or delay in the moulting process (120 h post-hatching) measured in each larva. Fitting of data to AFT model (endpoint ~ [GLY] + colony + [GLY] × colony) followed by Log-rank test for *post hoc* comparisons of simple effects. The number of larvae assessed for each colony (A-F) is shown in the table. Baseline proportions with different letters are significantly different among colonies. This table is a resume of Figs 1 and 2. LC, lethal concentration; SLC, sublethal concentration; EC, effective concentration; NOEL, no observable effect level in range assessed. For each colony, only GLY concentrations that have significant statistical differences with its baseline are reported.

Figure 1: Larval survival under chronic exposure to glyphosate for different honey bee colonies. Proportion of larval survival during exposure (5 days post-hatching) to contaminated food with GLY (range of concentrations assessed: 1.25±5.0 mg per litre). Survival curves are plotted with their confidence interval (95%) for each treatment of larvae reared *in vitro* and for each individual colony (A-F). The number of assessed larvae is shown in the graph. Fitting of data to AFT model (survival prop. ~ [GLY] + colony + [GLY] × colony) followed by a Log-rank test for *post hoc* comparisons of simple effects. The curves are plotted with different colours per treatment: *in vitro* control in blue and a yellow-red gradient for increasing GLY concentration treatments. The + indicates time points with censoring data. Different letters indicate significant differences among treatments in each colony.

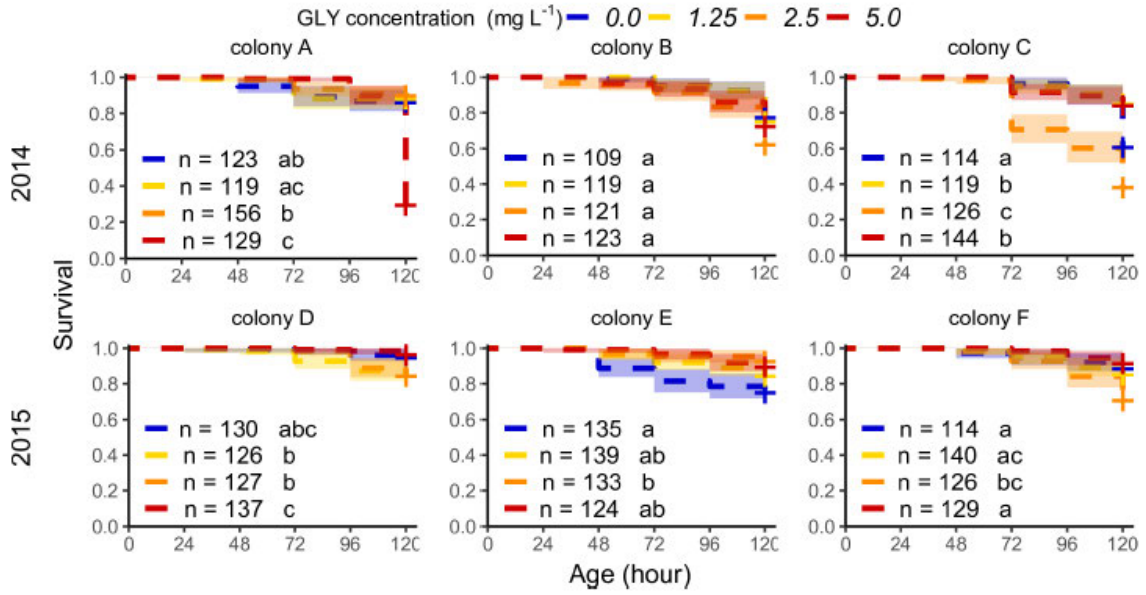
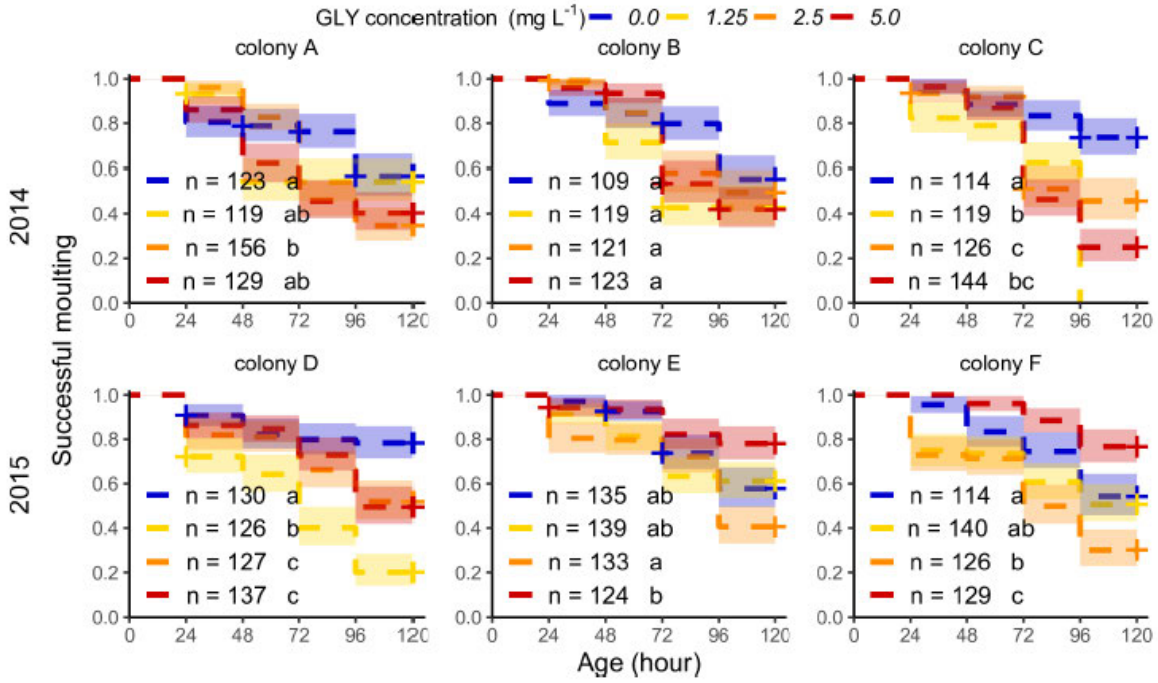


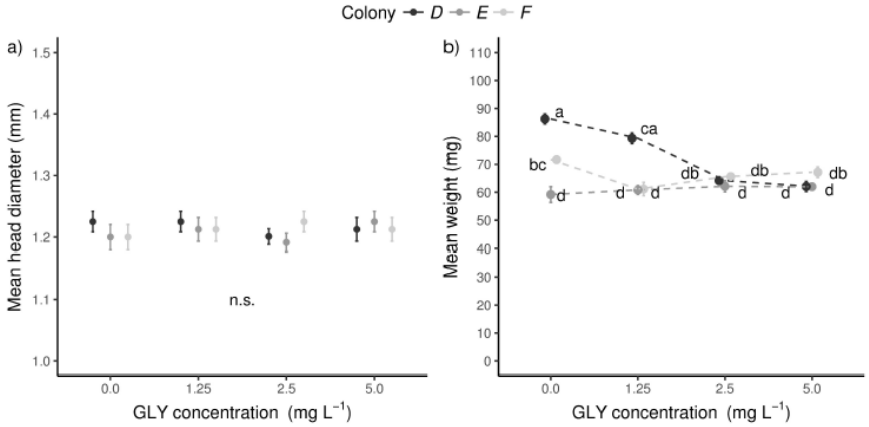
Figure 2: Larval moulting under chronic exposure to glyphosate for different honey bee colonies. Proportion of larvae without delay in moulting for each day during exposure (5 days post-hatching) to contaminated food with GLY (range of concentrations assessed: 1.25±5.0 mg per litre). Curves of successful moulting are plotted with their confidence interval (95%) for each treatment of larvae reared *in vitro* and for each individual colony (A-F). The number of assessed larvae for each treatment is shown in the graph. Fitting of data to AFT model (prop. of successful moulting ~ [GLY] + colony + [GLY] × colony) followed by a Log-rank test for *post hoc* comparisons of simple effects. The curves are plotted with different colours per treatment: blue for *in vitro* control and a yellow-red gradient for increasing GLY concentration. The + indicates time points with censoring data. Different letters indicate significant differences among treatments in each colony.



Changes in growth rate after GLY exposure

As GLY exposure led mainly to developmental adverse effects, the authors wished to investigate what effect it might have on the growth of those 5-day old larvae that accepted the total amount of contaminated food (same nutrition and dose) without notable adverse symptoms. To assess this, the authors measured the head diameter and weight in larvae from three colonies (D-F). Given the differences in the rearing conditions and nutrition, the authors also compared the growth of larvae from the same cohort between rearing contexts (in-hive and *in vitro*). Non-significant differences were detected in head diameter of sampled larvae among GLY concentrations (Kruskal Wallis test: head diameter ~ treatment. Treatment as a combination of [GLY] and colony, χ^2 (11) = 19.74, P = 0.05; Nemenyi test, d.f. = (12, 108), no significant multiple *post hoc* comparisons, Fig 3A). Nevertheless, both source colony and GLY exposure explain significant differences in weight with a significant interaction (GLM model: weight ~ [GLY] + colony + [GLY] × colony. F(1,108) = 20.54, P < 0.001, N = 120, *post-hoc* pairwise comparisons were performed with the Tukey test, see Fig 3B). Therefore, larvae exposed to GLY show a varying effect in growth among colonies. Colony E did not show an alteration in growth regardless of GLY concentration. Meanwhile, colony D showed a significant reduction of weight (around 27%) with a dose-dependent response in exposure to 2.5 and 5.0 mg/L of GLY. Furthermore, larvae from colony F showed a significantly lower weight (15%) for the lowest GLY concentration. No larva with delayed moult ate all offered food in any of the colonies (A-F). Experimental food during the feeding period was a mixture of different nutrients, mainly from commercial royal jelly. The pH was slightly acid (4.94 ± 0.05). The pH did not change (4.9 ± 0.18) in food incubated during five days under similar conditions to those of larvae reared *in vitro*, which indicates the stability of some of its properties. Nevertheless, food became slightly more acid when GLY is added in concentrations of 2.5 and 5.0 mg/L (GLMM model: pH ~ [GLY] + (time|replicate). [GLY] term: F (3, 87) = 24, P < 0.001. Variance structure: 0.5% through days and 6% among replicates.

Figure 3: Effect of GLY exposure on growth. Larvae exposed *in vitro* to GLY (1.25±5.0 mg of GLY per litre of food) without conspicuous adverse symptoms in larval development were sampled at 5-day of age from three colonies (D, E and F). We measured in each larva their (a) head diameter (mm) and (b) weight (mg). The number of larvae measured was 10 for each concentration per colony. Kruskal-Wallis test followed by Nemenyi *post hoc* comparisons was carried out to analyse morphometric data to compare among groups (n.s., no significant differences). GLM (weight ~ [GLY] + colony + [GLY] × colony) followed by Tukey *post hoc* comparisons was carried out to analyse simple effects in weight data. Groups with different letters have significantly different means.



Changes in gut gene expression of larvae exposed to GLY

In vitro rearing homogenizes environmental and nutritional factors among brood from different colonies. Therefore, the variability in effects is explained mainly by different genetic and/ or epigenetic factors among brood, associated with the response to a xenobiotic. The first internal barrier to contaminated food is the gut. Hence, the authors performed an exploratory analysis of the expression profile of immune/detoxifying genes. The authors sampled and dissected 5-day old larvae without adverse developmental effects from the colonies studied for morphological variables (D-F). The authors also analysed the expression profile of digestive enzymes and stress biomarkers genes to determine whether gut physiology had been disrupted as a result of GLY exposure. Finally, the authors compared gene expression between larvae reared in-hive and *in vitro* . On the one hand, expression levels of digestive enzymes and stressor biomarker genes were similar among colonies with low variability (in-hive: CVs 6 - 22%; *in vitro*: CVs 2 - 13%), suggesting that the *in vitro* rearing conditions do not disrupt gut physiology. Nevertheless, the authors did find high variability in the expression levels for some immune/detoxifying genes (in-hive: CVs 2 - 60%; *in vitro*: CVs 8 - 89%) among colonies in both contexts with a considerable trend to an increase in expression in the *in vitro* rearing. On the other hand, the authors analysed the gut responsiveness of larvae exposed to GLY. Due to the different baselines of gene expression among colonies in control groups, the authors relativized each expression profile to the control sample to compare the responsiveness patterns. Expression levels for digestive enzymes and stress biomarker genes seem similar between exposed and unexposed larvae. However, immune/detoxifying genes are regulated by GLY or its subproducts as a consequence of the exposure, with a differential response among colonies. Colony D showed a general downregulation of immune/detoxifying genes. Colonies E and F showed upregulation regardless of GLY concentration, especially in genes *CYP6AS2* and *CYP9Q3*, in both colonies. Colony F showed an upregulation of the immune gene *Abaecin* and a downregulation of *CYP6AS4* with a dose-dependent response opposite to that of colony E. The clustering method showed a similarity between expression baselines of colonies E and F when using their complete gene expression profiles. All samples from exposed larvae were grouped separately from control larvae. In addition, the expression profiles of the samples exposed to the same concentrations of GLY in colonies E and F were grouped, indicating a similar response for both colonies. Thus, the variability in the regulation of immune/detoxifying genes could explain the variability in tolerance to GLY exposure.

Conclusion

The results showed that brood fed with food containing GLY traces (1.25±5.0 mg per litre of food) had a higher proportion of larvae with delayed moulting and reduced weight. The assessment also indicates a non-monotonic dose-response and variability in the effects among colonies. Differences in genetic diversity could explain the variation in susceptibility to GLY. Accordingly, the transcription of immune/detoxifying genes in the guts of larvae exposed to GLY was variably regulated among the colonies studied. Consequently, under laboratory conditions, the response of honey bees to GLY

indicates that it is a stressor that affects larval development depending on individual and colony susceptibility.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The method of exposure used for the bees were not described. Endpoints presented are not relatable to an EU level ecotoxicological regulatory risk assessment for the glyphosate EU renewal.

Assessment and conclusion by RMS:

The study aimed to assess the effects of glyphosate on larval development under chronic exposure during in vitro rearing. The study exposed brood with food containing glyphosate (1.25±5.0 mg per litre of food) and investigated larval survival among other paramaeters such as delayed moulting, reduced weight, their variability among colonies. The transcription of immune/detoxifying genes in the guts of larvae exposed to GLY was studied.
The brood received a total dose of glyphosate of 137.5, 275 and 550 ng a.e. per treatment.
RMS considers the parameters investigated of low relevance except for brood survival.
However RMS does not agree with the conclusion of the study as the performance of the control was very variable among colonies. OECD GD 239 requires that in the control plate(s), cumulative larval mortality from D3 to D8 should be ≤15% across all replicates. RMS notes that larval mortality was above 15% in at least 3 colonies (out of 6, i.e. in colonies B, C and E). Besides, the study authors noted a 21% reduction in weight of in vitro reared larvae with respect to larvae reared in-hive, which was (according to them) evidence of malnutrition. They note the malnutrition state and the absence of social immunity of larvae reared in vitro cannot be ignored.
Overall the condition of the tested larvae in uncertain.
There is uncertainty on the dosing. Pure glyphosate was used but no analytical verification was reported (A sample of the solution used to prepare the diet should have been made for analytical determination of the actual concentration of the test chemical). More importantly, as noted by the study authors, larvae with delayed development did not eat all the food offered (in any of the colonies), regardless of whether they have been exposed to glyphosate or not. The study authors noted that the moulting process depends on growth rate, which is linked to feeding, that is homogenized in the in vitro assessment. As food is a constraint for the immunity of brood, its properties are important under these conditions. According to the study authors, the acidification of rearing food by glyphosate has unknown consequences (food became slightly more acid in concentrations of 2.5 and 5.0 mg L^{-1}).
No toxic reference was used to ensure that the test system and conditions are responsive and reliable.
Besides RMS does not agree with the conclusion of the study authors that weight was reduced. The largest difference was found on control larvae (based on 3 colonies). Larvae exposed to glyphosate did not show weight difference (except for one colony out of three and at lowest dose).
No dose effect relationship was observed.
No reliable endpoint can be derived from this study.
The study is considered relevant but not reliable for regulatory risk assessment purpose.

Data point:	CA 8.3.1.2
Report author	Blot N. *et al.*
Report year	2019
Report title	Glyphosate, but not its metabolite AMPA, alters the honeybee gut microbiota.

Document Source	PloS one (2019), Vol. 14, No. 4, Article No. e0215466
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The honeybee (*Apis mellifera*) through the foraging and storing of contaminated resources, are exposed to xenobiotics. As ingested glyphosate and aminomethylphosphonic acid (AMPA, main metabolite of glyphosate) are directly in contact with the honeybee gut microbiota, this study used quantitative PCR to test whether they could induce significant changes in the relative abundance of the major gut bacterial taxa.
Glyphosate induced a strong decrease in *Snodgrassella alvi*, a partial decrease of a *Gilliamella apicola* and an increase in *Lactobacillus* spp. abundances. *In vitro*, glyphosate reduced the growth of *S. alvi* and *G. apicola* but not *Lactobacillus kunkeei*. Although being no bee killer, the study confirmed that glyphosate can have sublethal effects on the honeybee microbiota. To test whether such imbalanced microbiota could favor pathogen development, honeybees were exposed to glyphosate and to spores of the intestinal parasite *Nosema ceranae*. Glyphosate did not significantly enhance the effect of the parasite infection. Concerning AMPA, while it could reduce the growth of *G. apicola in vitro*, it did not induce any significant change in the honeybee microbiota, suggesting that glyphosate is the active component modifying the gut communities.

Materials and methods

Honeybee artificial rearing
Experiments were performed on interior workers collected on brood frames from five colonies of the same apiary in Clermont-Ferrand, France.

Each of the five colonies represented an independent replicate. Workers were divided into cohorts of 70 individuals in cages, incubated and fed with sugar syrup. In treated conditions, the syrup was supplemented with pure glyphosate (Interchim SS-7701) and/or AMPA (Sigma-Aldrich 324817).

A first experiment was performed on overwintering honeybees (February 2018) that were submitted to (i) no treatment, (ii) a chronic exposure to 1.5 mM glyphosate in their sugar syrup (or 0.21 g/kg according to syrup density), (iii) an infection with spores of the parasite *Nosema. ceranae*, (iv) an infection by *N. ceranae* and a chronic exposure to 1.5 mM glyphosate.

Chronic exposure to herbicides was performed by feeding honeybees *ad libitum* with sugar syrup containing pesticides. The absence of *N. ceranae* and *Nosema apis* in the colonies was checked by PCR on 20 honeybees before experiment initiation. *N. ceranae* infection was performed one day before experiment initiation, by collectively infecting cohorts of 70 honeybees with a mean of 150 000 spores of the parasite per bee in 2.5 mL of syrup until complete consumption. *N. ceranae* spores were obtained

according to Vidau *et al*.. Dead bees were removed daily. Feeders were replaced and weighed daily to measure sucrose consumption. The surprisingly strong effect observed on the microbiota by glyphosate led to repeat the experiment on cohorts of summer honeybees (June 2018) from the same five colonies, but with more concentrations and contaminants: (i) no treatment and chronic exposures to (ii) 1.5 mM (0.21 g/kg) glyphosate, (iii) 7.5 mM (1.08 g/kg) glyphosate, (iv) 1.5 mM (0.14 g/kg) AMPA, (v) 7.5 mM (0.70 g/kg) AMPA, (vi) 1.5 mM glyphosate and 1.5 mM AMPA, (vii) 7.5 mM glyphosate and 7.5 mM AMPA.

The chosen concentrations of glyphosate are above most of the ones measured in hives matrices in other studies. However they are related to the mean concentrations found in foraged pollen one day (0.47 g/kg) and four days (0.16 g/kg) following an experimental semi-field treatment. The experiments would thus be related to an unusually persistent exposure to glyphosate.

Gut DNA purification and PCR quantification of bacterial taxa
Honeybees were sacrificed 15 days after the experiment initiation. The presence or absence of *Nosema* spores in individuals was checked by microscopy (x400). For each experimental condition and replicate, the full digestive tracts of 8 honeybees were dissected on ice, pooled and stored at -80˚C. The total DNA of pooled guts was purified as described in Engel *et al*.. QPCR analyses were performed.

Specificity of reactions was checked by analyzing the melting curves of the amplified products. Statistical analyses were performed using PAST software. Non-normal data gave no significant difference using paired Wilcoxon signed rank or Mann & Whitney tests. Paired t-test was performed for normal data and differences were considered significant for *p*-values below 0.05. When a difference was significant, a similar positive or negative effect was observed in all of the five colony replicates.

Isolation and growth of gut bacterial strains
Single guts from nine interior honeybees were dissected and homogenized in 800 µL of 1X Phosphate-Buffered Saline. Colonies were picked up and isolated onto the same medium several times until axenic liquid cultures were obtained, as verified by re-plating and microscopic observation of Gram-stained bacteria. Thirteen strains were isolated and their genomic DNA was extracted. Cultures of seven selected isolates were diluted 50 times in fresh HIB medium, but supplemented MRS medium for *Lactobacillus* strains. 750 µL of the dilution were deposited in wells of a 24-well plate containing various quantities of glyphosate and AMPA. Six independent cultures were performed for each herbicide concentration. After 48h of incubation as above, the optical density (OD) of the cultures were measured at 600 nm, using sterile medium as a blank. For statistical analyses, Student t-test was performed for normal data otherwise the Mann & Whitney test was performed. Differences were considered significant for *p*-values below 0.05.

Results

Survival analyses demonstrated that glyphosate but also AMPA, alone or together, did not induce any significant decrease in honeybee survival, compared to control, whatever the season. In contrast a significant decrease of survival was observed in *Nosema*-infected bees as previously observed. No synergistic effect was observed between glyphosate and *N. ceranae* or between glyphosate and AMPA. The constant decrease on survival in summer could be associated with the shorter lifespan of honeybees during the beekeeping season. The data confirmed previous studies showing that glyphosate is no bee killer and showed that it is also true for AMPA. Thus, whatever the observed effects on the microbiota, they ought to be sublethal.

The daily sugar consumption was affected neither by glyphosate, nor AMPA, nor *N. ceranae*. The oral and topic LD_{50} of glyphosate are far greater than 0.1 mg of active ingredient per bee. Zhu *et al*. estimated the topic LD_{50} as 3.5 × 1031 µg/bee, *i.e*. about half the mass of the moon of glyphosate per bee. The daily and cumulative consumptions of glyphosate were thus clearly far below lethality.

The honeybee gut microbiota can be altered by fungicides and acaricides. The authors tested whether it can be affected by the herbicide glyphosate or by its metabolite AMPA. The relative abundance of the major bacterial taxa of the microbiota, normalized to the total bacterial DNA, was assessed by QPCR after 15 days of chronic exposure. Two-way ANOVA showed significant interactions neither between season and glyphosate treatment, nor between glyphosate and *N. ceranae*, nor between glyphosate and AMPA. Multivariate analyses suggested that season and glyphosate, but not AMPA were the two most important components explaining the observed variances, with *S. alvi* as the major factor explaining the effect of glyphosate.

To test whether a glyphosate exposure could increase the effect of an infection by the intestinal parasite *N. ceranae*, overwintering honeybees were co-exposed to spores of the parasite and to glyphosate. No stronger effect was observed on mortality, sugar consumption and bacterial abundances when both the parasite and the herbicide were applied.

In order to test whether honeybee gut bacteria were sensitive to glyphosate and AMPA, strains were isolated from the guts of nine untreated honeybees. Thirteen strains were isolated and identified by the cloning and sequencing of their SSU RNA encoding gene. Seven strains were selected to test the sensitivity of their growth in the presence of increasing concentrations of glyphosate and AMPA. The growth of *S. alvi* was very significantly reduced in the presence of glyphosate but not in the presence of AMPA. Moreover the impairment of *S. alvi* growth seemed dose-dependent. The isolated strain of *G. apicola* was also sensitive to glyphosate, to a lesser extent. The growth of *G. apicola* was also significantly reduced in the presence of AMPA, and completely impaired in the presence of 5 mM AMPA. The *Bifidobacterium* sp. strain was also sensitive to glyphosate but not to AMPA. In contrast the isolated strains of *Lactobacillus*, and *Staphylococcus* were more resistant to the herbicide and its metabolite.

Conclusion

Glyphosate has sublethal effects on the honeybee gut microbiota, changing the abundance of major bacterial taxa, especially by affecting the growth of *S. alvi*. The consequences of such microbial disturbance are unclear: is the honeybee able to cope with glyphosate and preserve the gut homeostasis or will future data demonstrate that these changes are linked with a functional dysbiosis?

An increasing amount of data proves that the honeybee microbiota takes its part in the response to stressors, showing that the whole holobiont, *i.e*. the honeybee and its microbial communities, should be considered when challenging environmental constraints.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: In this publication, experiments were conducted with a dose (10x increased) and an exposure for a longer period than is expected to occur from field exposure. Results indicated no effect on survival but some effect on profile of gut microbes. AMPA did not affect profile which could be due to AMPA does not inhibit EPSPS.
Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear.

Further point of clarification
The findings are not relatable to an EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal.

Assessment and conclusion by RMS:

Results indicated that glyphosate had some effect on honeybee microbiota but not AMPA.
The present study states that its results confirm those of Motta et al. 2018 by showing that glyphosate, although not lethal for the honeybee, can alter its gut microbiota. (Motta et al. 2018 was also assessed by RMS.) It states that "*in relative abundance, a strong decrease in of S. alvi and an increase in Lactobacillus spp. were observed in response to glyphosate, which was consistent with the data of Motta et al. 2018 in newly emerged and interior bees. In contrast, opposite results were observed on G. apicola. In absolute abundance, Motta et al. 2018 showed that glyphosate induced a reduction in the total bacterial content of the honeybee gut microbiota, as well as a decrease in S. alvi, Lactobacillus spp. and Bifidobacterium spp*.".

Several experiments were conducted in the study to show that exposure of adult bees might impact the gut microbiome leading to a greater susceptibility to pathogen infections.
On this aspect, the study is not directly relevant for the risk assessment but still relevant for the investigation of "other types of effects" not currently covered by the risk assessment scheme. It is considered by RMS as "additionnal data". RMS notes the absence of clear conceptual link between effects on the honeybee microbiota and the specific protection goals for bees (SPG). It is agreed that it may play a role in the colony/population health, but such link is not immediate in conceptual terms and not quantifiable. No data can be used for the standard risk assessment.

However only graphics are reported, no biological data reported. Besides, as noted for Motta et al, 2018, some shortcomings render the study lowly reliable (e.g. small sample sizes, influence of age on gut microbiome, lack of confirmation of the levels of actual glyphosate exposure, bees diets had no source of amino acids, etc.).
Overall, the study is considered relevant but not reliable.

Data point:	CA 8.3.1.2
Report author	Motta E. V. S. *et al.*
Report year	2018
Report title	Glyphosate perturbs the gut microbiota of honey bees.
Document Source	Proceedings of the National Academy of Sciences of the United States of America (2018), Vol. 115, No. 41, pp. 10305-10310
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevance cannot be determined (EFSA GD Point 5.4.1 - category C)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Glyphosate, the primary herbicide used globally for weed control, targets the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme in the shikimate pathway found in plants and some microorganisms. Thus, glyphosate may affect bacterial symbionts of animals living near agricultural sites, including pollinators such as bees. The honey bee gut microbiota is dominated by eight bacterial species that promote weight gain and reduce pathogen susceptibility. The gene encoding EPSPS is present in almost all sequenced genomes of bee gut bacteria, indicating that they are potentially susceptible to glyphosate. The present study aimed to demonstrate that the relative and absolute abundances of dominant gut microbiota species are decreased in bees exposed to glyphosate at concentrations documented in the environment.

Materials and methods

Effects of Glyphosate on the Honey Bee Gut Microbiome
Hundreds of adult worker bees were collected from a single hive, treated with either 5 mg/L glyphosate (G-5), 10 mg/L glyphosate (G-10) or sterile sucrose syrup (control) for 5 d, and returned to their original hive. Bees were marked on the thorax with paint to make them distinguishable in the hive. Glyphosate concentrations were chosen to mimic environmental levels, which typically range between 1.4 and 7.6 mg/L (Herbert LT et al, 2014), and may be encountered by bees foraging at flowering weeds. To determine the effects of glyphosate on the size and composition of the gut microbiome, 15 bees were sampled from each group before re-introduction to the hive (day 0) and post-reintroduction (day 3), and relative and absolute abundances of gut bacteria were assessed using deep amplicon sequencing of the V4 region of the bacterial 16S rRNA gene and quantitative PCR (qPCR).
DNA samples from the first experiment were submitted for Illumina sequencing at the Genomic Sequencing and Analysis Facility (GSAF) at UT Austin. Illumina sequence reads were processed using QIIME 1.9.1 (Caporaso JG, et al, 2010).

Effects of Glyphosate on Early Gut Colonization and Susceptibility to Serratia Infection
Hundreds of late-stage pupae were removed from brood frames and allowed to emerge under sterile conditions in laboratory. (Experiment A) NEWs were exposed to bee gut homogenate for 5 d, then hand fed 1 mM glyphosate or sterile sugar syrup on 2 alternate days. Fifteen bees from each group were sampled 2 d after the last hand feeding. DNA was extracted from dissected guts, used as template for qPCR analyses, and submitted for Illumina sequencing at the GSAF, UT Austin. (Experiment B) NEWs were exposed to a bee gut homogenate or sterile sucrose syrup. Half of the subgroups was exposed to the opportunistic pathogen *S. marcescens* kz19, whereas the other half was used as controls. Bees were exposed to similar amounts of glyphosate (~1.7 μg) in experiments A and B.

S. alvi Colonization During Glyphosate Exposure
NEWs were hand fed 5 μL sucrose syrup containing ~10^5 cells of *S. alvi* wkB2 or wkB339 or sterile sucrose syrup as control. Each group was divided into two subgroups and treated with 0.1 mM glyphosate or sterile sucrose syrup for 3 d immediately following bacterial exposure. Eight bees were sampled from each subgroup at days 1 and 3, and DNA was extracted from dissected guts. *S. alvi*-specific primers (Martinson VG, et al, 2012) were used to amplify total copies of 16S rDNA of each sample by qPCR.

In Vitro Experiments with Bee Gut Bacterial Strains
Honey bee and bumble bee gut bacterial strains were cultured in InsectaGro or MRS broth in the presence or absence of 10 mM glyphosate in a 96-well plate and incubated in a plate reader at 35 °C and 5% CO_2 for 48 h. Optical density was measured at 600 nm every 6 h. Experiments were performed in triplicate.

Plasmid Construction and Transformation
The *aroA*, *yhhS*, and *tetC* genes from various bacterial strains were PCR amplified and cloned into the arabinose-inducible pBAD30 vector (Guzman L-M, et al, 1995) by Gibson assembly (Gibson DG et al,

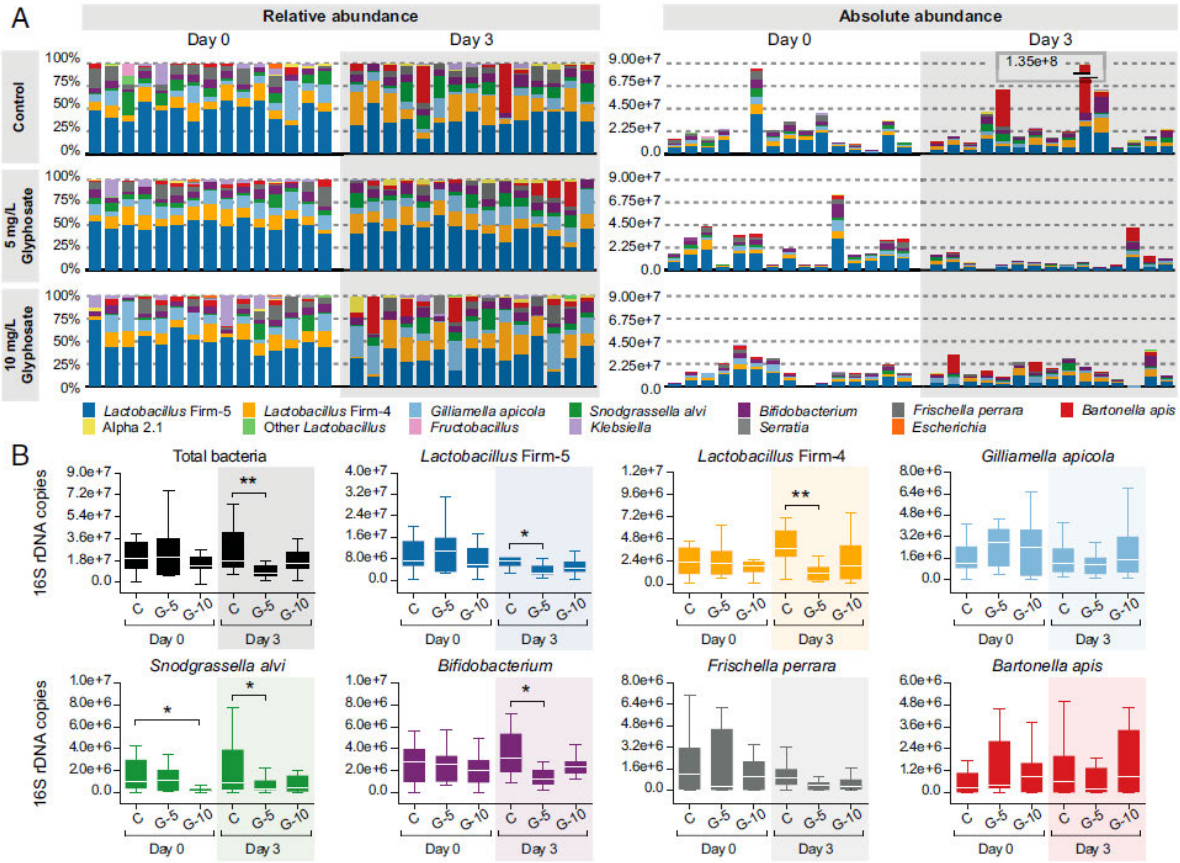
2009) and then used to transform *E. coli* strain BW25113 or a derivative lacking the *aroA* gene by electroporation.

Growth Rate Analysis of Transformed E. coli
Transformed *E. coli* cells were cultured in duplicate in 24-well plates containing M9 minimal medium (Joyce AR et al, 2006) with appropriate antibiotics, varying concentrations of glyphosate, and varying concentrations of arabinose. The plates were incubated in a plate reader at 37 °C for 24 - 96 h. Optical density was measured at 600 nm every hour.

Results

At day 0, glyphosate exposure had little effect on the bee gut microbiome size, but the absolute and relative abundances of the core species, *S. alvi*, were significantly lower in the G-10 group. The effects of glyphosate exposure on the bee gut microbiome were more prominent at day 3, after treated bees were returned to the hive. The total number of gut bacteria decreased for both treatment groups, relative to control, but this drop was significant only for the G-5 group, which also exhibited more severe compositional shifts (Fig. 1). The absolute abundances of four dominant gut bacteria, *S. alvi, Bifidobacterium, Lactobacillus* Firm-4 and Firm-5 were decreased (Fig. 1), and the relative abundance of *G. apicola* increased in the G-5 group. Surprisingly, only *Lactobacillus* Firm-5 decreased in absolute abundance in the G-10 group (Fig. 1). This experiment was repeated using bees from a different hive and season, and similar trends were observed. As in the first experiment, significant reductions in abundance were observed for *S. alvi* in bees treated with glyphosate.

Figure 1. Changes in gut microbiota composition following glyphosate exposure of honey bees with established gut communities. (A) Stacked column graph showing the relative and absolute abundances of gut bacterial species in control bees and bees treated with 5 mg/L or 10 mg/L glyphosate at post treatment days 0 and 3. Each column represents one bee. (B) Boxplots of bacterial 16S rDNA copies for control (C) and glyphosate-treated (G-5 and G-10) bees at post-treatment days 0 and 3.



Glyphosate Affects Early Gut Bacterial Colonization

Assessment of gut microbiomes, identified all eight core gut taxa in both control and treatment groups, showing that glyphosate does not eliminate colonization by any core member. Average total bacterial abundance was slightly lower in glyphosate-treated bees, but this was not statistically significant. *S. alvi* was the most strongly affected member of the gut microbiota and decreased in both absolute and relative abundance, while *Lactobacillus* Firm-4 increased in relative abundance. Thus, glyphosate exposure during early development of the gut community can interfere with normal colonization by altering the abundance of beneficial bacterial species.

The authors analyzed changes in bacterial abundance after glyphosate exposure by extracting both DNA and RNA from the guts of treatment and control bees in a second colonization experiment. They included a positive control group, in which bees were exposed to tylosin, an antibiotic used in beekeeping.

Glyphosate Exposure Makes Young Worker Bees More Susceptible to Serratia

To determine whether glyphosate-induced perturbation of microbiota colonization affects host health, the authors measured the susceptibility of glyphosate-treated bees to an opportunistic bacterial pathogen. NEWs were exposed to glyphosate in the stage of acquiring their normal microbial community. After 5 d of treatment, bees were challenged with *Serratia marcescens* kz19, an opportunistic pathogen commonly detected at very low frequencies in the bee gut.

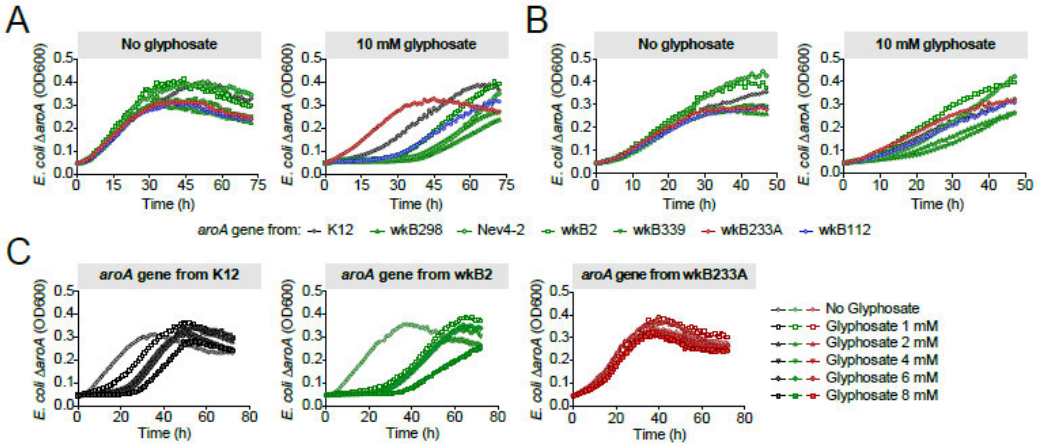
For bees lacking gut microbiota, *Serratia* challenge resulted in increased mortality relative to that observed for bees with a conventional gut microbiota, regardless of glyphosate exposure. For bees with

a conventional gut microbiota, glyphosate treatment resulted in increased mortality after *Serratia* challenge. To determine whether this increased mortality was attributable to the effects of glyphosate on the gut microbiota or to direct effects of glyphosate on bees, the authors included control groups not challenged with *Serratia*. In bees exposed to glyphosate, but not challenged with *Serratia*, survival rates were not significantly affected by glyphosate and were much higher than in the *Serratia*-challenged groups, demonstrating that a direct effect of glyphosate on bees is not the basis of the high mortality of glyphosate-exposed, pathogen-challenged bees.
The results show that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens and that *S. alvi* is the bacterial species most negatively affected by glyphosate exposure. By itself, *S. alvi* appears to give some protection, but not as much as the whole gut microbiota.

The Bee Gut Contains Bacterial Species with both Sensitive and Insensitive Types of EPSPS
Bacterial EPSPS exists as two main types, corresponding to two phylogenetic clusters, that differ in sensitivity to glyphosate: Class I is naturally sensitive, whereas class II is insensitive. Several bee gut-associated bacterial strains isolated from honey bees and bumble bees were cultured *in vitro* in the presence or absence of a high dose of glyphosate. Most *S. alvi* and *G. apicola* strains tested, which contain a class I EPSPS, either do not grow or have a delay in growth when cultured in the presence of glyphosate; no such effect is observed for strains containing a class II EPSPS, *Lactobacillus* Firm-4 and *B. apis* . However, *S. alvi* strains wkB2 and wkB298, despite containing a class I EPSPS, grow as well in the presence of glyphosate as in its absence, with no initial delay in growth. The authors looked for potential single-site mutations in the EPSPS active site of these strains, which is known to confer tolerance to glyphosate (Eschenburg S, et al, 2002), but no mutations were observed, indicating that the resistance in these *S. alvi* strains results from other mechanisms.
Bee gut bacterial strains having a glyphosate susceptible EPSPS are predicted to drop in abundance following exposure, as observed for *S. alvi* and *Bifidobacterium* in the hive experiments. *Lactobacillus* Firm-4, which encodes a class II EPSPS, and Firm-5, which does not contain the target enzyme of glyphosate, also had their abundances reduced in the hive experiment, which was not expected.
This may be explained by the fact that these strains lack the aromatic amino acid biosynthetic pathway (18), relying on uptake of aromatic amino acids released by other bacterial species, such as S. alvi, in the bee gut environment. The increase in G. apicola relative abundance was unpredicted, but was also observed in a previous study on microbial community responses to antibiotic perturbation.

Glyphosate Resistance Is Independent of EPSPS Class in Some Bee Gut Strains
To understand the mechanism that prevents some bee gut bacterial strains from growing in the presence of glyphosate, we complemented *E. coli ΔaroA* with *aroA* genes cloned from bee gut bacterial strains as well as with the *E. coli* K12 *aroA*, which is known to be sensitive to glyphosate.
Transformants carrying the *aroA* gene from *S. alvi, G. apicola,* and *B. apis* were able to grow in minimal media at a similar rate to the transformant carrying the *aroA* gene from *E. coli*. The addition of 10 mM glyphosate to the media resulted in a delay in growth of ∼48–72 h for transformants carrying the *aroA* gene from all *S. alvi* and *G. apicola* strains tested. This is expected if glyphosate binds to a susceptible EPSPS, blocking the shikimate pathway and preventing bacterial growth until the concentration of PEP or EPSPS exceeds that of glyphosate, allowing the transformants to resume growth. On the other hand, the transformant carrying the *aroA* gene from *B. apis* did not exhibit the growth delay in the presence of glyphosate, as predicted since this *aroA* version encodes an insensitive class II EPSPS. Moreover, the addition of increased concentrations of arabinose in the media or reduction in glyphosate concentration sped up the growth of all transformant strains (Fig. 2), which corroborates the reversible mechanism of EPSPS inhibition by glyphosate.

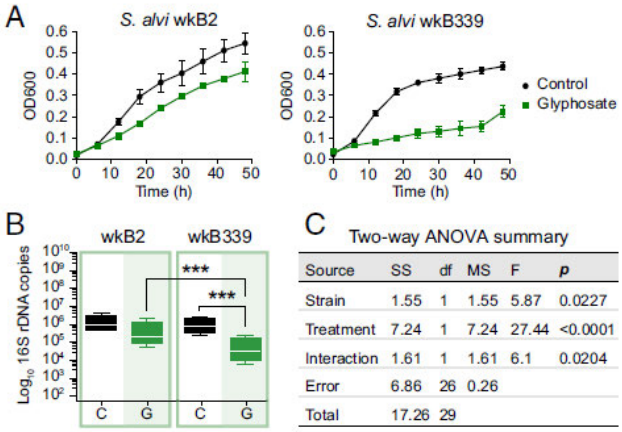
Figure 2. Growth of *E. coli ΔaroA* BW25113 expressing the *aroA* gene from different bee-associated bacterial strains cultured in minimal media in the presence or absence of 10 mM glyphosate, and A) 0.001% or B) 0.01% arabinose. C) Transformants were cultured in minimal media in the presence of different glyphosate concentrations ranging from 1 to 8 mM, and no arabinose. *B. apis* in red, *E. coli* in black, *G. apicola* in blue, and *S. alvi* in green.



S. alvi Strains May Vary in Sensitivity to Glyphosate in Vivo

The phylogenetic analysis and cloning experiments demonstrated that, despite displaying variable susceptibility to glyphosate in vitro, all *S. alvi* strains possess a glyphosate-sensitive class I EPSPS. Therefore, the authors investigated whether this variation in susceptibility occurs *in vivo*. NEWs were monoinoculated with two different *S. alvi* strains: wkB2, which grows in the presence of high concentrations of glyphosate, or wkB339, which exhibits a delay in growth in the presence of high concentrations of glyphosate. Bees were hand fed bacterial suspensions to inoculate with a control number of *S. alvi* cells, exposed to glyphosate for 3 d, and sampled at days 1 and 3 during exposure. Both *S. alvi* wkB2 and *S. alvi* wkB339 increased in abundance between days 1 and 3 in unexposed bees. Based on qPCR estimates of *S. alvi* abundance on day 3, glyphosate exposure had a negative effect on growth of both strains [two-way analysis of variance (ANOVA), treatment effect, $P < 0.0001$]. *S. alvi* wkB339 was more affected by glyphosate exposure, based on significant interaction between strain and treatment (two-way ANOVA, $P < 0.0204$). Correspondingly, the absolute abundance of the glyphosate-sensitive strain, wkB339, was significantly lower in glyphosate-treated bees compared with controls (Tukey's test, $P < 0.001$) or wkB2-treated bees (Tukey's test, $P < 0.001$) (Fig. 3 B and C). Potentially, strain differences in glyphosate sensitivity may contribute to the observed variation in the overall decrease in *S. alvi* abundance when bees with their native gut microbiota are exposed to glyphosate.

Figure 3. Variation in *S. alvi* strain sensitivity to glyphosate. (A) Growth curves of *S. alvi* wkB2 and wkB339 cultured in InsectaGro media in the presence or absence of 10 mM glyphosate. Experiment was performed in triplicate, and each data point represents the average optical density (600 nm, with SD bars). (B) Boxplots of *S. alvi* wkB2 and wkB339 abundances in bees exposed or not to 0.1 mM glyphosate for 3 d estimated by qPCR. Box-and-whisker plots show high, low, and median values, with lower and upper edges of each box denoting first and third quartiles, respectively. ***P < 0.001, two-way ANOVA with Tukey's correction for multiple comparisons. (C) Two-way ANOVA for effects of *S. alvi* strain and glyphosate treatment.



C Two-way ANOVA summary

Source	SS	df	MS	F	p
Strain	1.55	1	1.55	5.87	0.0227
Treatment	7.24	1	7.24	27.44	<0.0001
Interaction	1.61	1	1.61	6.1	0.0204
Error	6.86	26	0.26		
Total	17.26	29			

Conclusion

The authors found that glyphosate affects the bee gut microbiota composition and that bacterial species and strains within this community vary in susceptibility to glyphosate. The results also suggest that establishment of a normal microbial community is crucial for protection against opportunistic pathogens of honey bees. Furthermore, our results highlight one potential mechanism by which glyphosate affects bee health.
While some species in the bee gut can tolerate high concentrations of glyphosate due to the presence of a class II EPSPS enzyme, others are sensitive due to the presence of a class I EPSPS. A consistent effect of glyphosate on the bee gut microbiota was a negative impact on growth of *S. alvi*, which possesses a sensitive EPSPS. However, some strains of *S. alvi* may tolerate glyphosate through an as yet unknown mechanism. Since bee gut symbionts affect bee development, nutrition, and defense against natural enemies, perturbations of these gut communities may be a factor making bees more susceptible to environmental stressors including poor nutrition and pathogens.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case c) Relevance cannot be determined: This papers describes exposure of bees to glyphosate and its impact on gut microbiota. Potential effects to gut microbes are not part of the EU risk assessments. Suitable scientific approaches to assess effects are not specified, thus relevance of the effects remained unclear.

Further point of clarification
The findings are not relatable to an EU level ecotoxicological regulatory risk assessment / glyphosate EU renewal.

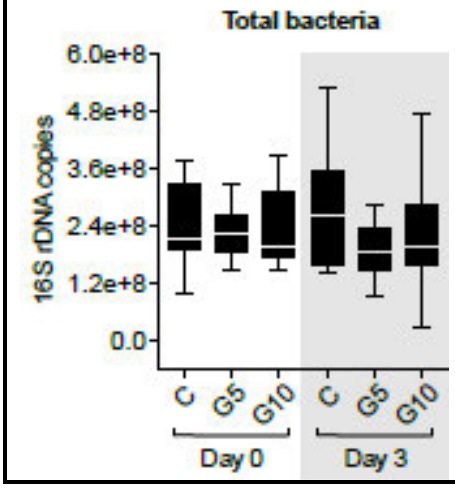
Assessment and conclusion by RMS:

This study states that glyphosate had some effect on honeybee microbiota.
RMS notes the absence of clear conceptual link between effects on the honeybee microbiota and the specific protection goals for bees (SPG). It is agreed that it may play a role in the colony/population health, but such link is not immediate in conceptual terms and not quantifiable.
The only bee mortality reported were related to an exposure to an opportunistic bacterial pathogen Serratia marcescens kz19 (with and without glyphosate). This study states that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens. The effect of such synergistic effects are however not covered by the current risk assessment scheme.

Several experiments were performed in the study and the details of those studies were mainly presented in the Supplementary Information (not provided by the applicant but could be retrieved by RMS).

Results
Hive experiments
Two hive experiments were performed, in autumn and spring. In each experiment, the same procedure was followed: 2000 adult bees were collected from a single hive, separated into 3 groups (control, 5 and 10 mg glyphosate/L), and placed into cup cages (40 bees per cup cage, totaling 16 cup cages per group). The bees were exposed to glyphosate during 5 days. Then 15 bees from each group were sampled (Day 0), and 600 bees from each group were returned to the hive (it is not reported how these were chosen, nor what was done with the other 1385 bees). At Day 3 post exposure (Day 3), 15 marked bees from each group were sampled from the hive. Fewer than 20% of returned bees were recovered from each group at Day 3. Relative and absolute abundances of gut bacteria were assessed. The exposure levels chosen in the hive experiment (5 and 10 mg glyphosate/L) are claimed to mimic those expected in fields, i.e. 1.4 – 7.6 mg glyphosate/L (a reference is made to another article). The relevance of these exposure levels is not established.

In the first hive experiment, at d0 glyphosate exposure had little effect on the bee gut microbiome size (total bacteria number). The authors claim that the effects of glyphosate exposure on the bee gut microbiome were more prominent at day 3, after treated bees were returned to the hive. However, although the effects of G-5 treatment were statistically significant relative to the control, the abundance of bacteria in the control and the G-10 treatment were similar at d3. Therefore, the biological relevance of the results is not fully justified at d3. In the second hive experiment, significant differences were not found between the control and the treatments at either d0 or d3 (see graphic below).

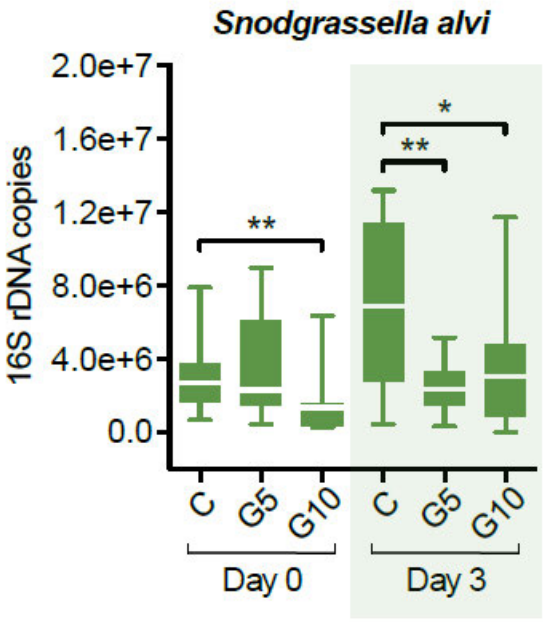


Numbers of total bacterial 16S rDNA copies for control (C) and glyphosate-treated (G-5 and G-10) bees at post-treatment Days 0 and 3 (n = 13 for each group and time point) in the second hive experiment.

The report states that absolute abundances of *Lactobacillus Firm-4* (among others) were decreased in G-5 and G-10. However RMS notes that relative and absolute abundances of *Lactobacillus Firm-4* increased in control group from d0 to d3. This indicates that these parameters were not steady and a shift may not be explained by dose treatment only.

At d0, the relative and absolute abundances of the core species, *S. alvi*, were significantly lower in the G-10 group at d0, while this reduction was not obvious at d3 (Fig 1, A), this was not explained.

In the second hive experiment, a significant reduction in absolute abundance of *S. alvi* was observed at d0 and d3 in the G-5 and G-10 treatment (although no clear concentration response could be established). (see graphic below). RMS notes that absolute abundance was not homogeneous among bees in different groups (including control at Day 3) and the number of bees was low (13).



Numbers of 16S rDNA copies for *S. alvi* for control (C) and glyphosate-treated (G-5 and G-10) bees at post-treatment Days 0 and 3 (n = 13 for each group and time point) in the second hive experiment.

Several drawbacks in this study are noted:

- the sample size was 15 bees per treatment (2 sampling times = 30 bees), which is relatively low to provide an understanding of natural background variability versus actual effects,
- bees from only 2 hives were sampled (spring and autumn),
- the rearing conditions of bees were not reported, which might have influence on the results and is of importance to judge the health of bees,
- all bees (for the 3 groups) were taken from and returned to the same hive – the test groups were therefore not isolated from each other and thus it is unknown whether a transfer of glyphosate and bacteria might have occurred among bees,
- variations in gut bacteria are common in different hives, but also between different individuals, but there was no way to compare this information in the experiments, nor any information provided by the study authors regarding this,
- the microbiome composition is influenced by age and since the age of the bees in the experiment was not reported, and it is possible that bees of different ages were used, it is not clear whether it is appropriate to compare between the groups in the experiment,
- the exact amount of consumed sucrose syrup is not reported and the actual doses of glyphosate per bee per day therefore cannot be calculated,

- the bees were not fed with pollen (a source of proteins and enzymes for nurse bees), which may have influenced the results since many microbes are dependent upon amino acids for survival.

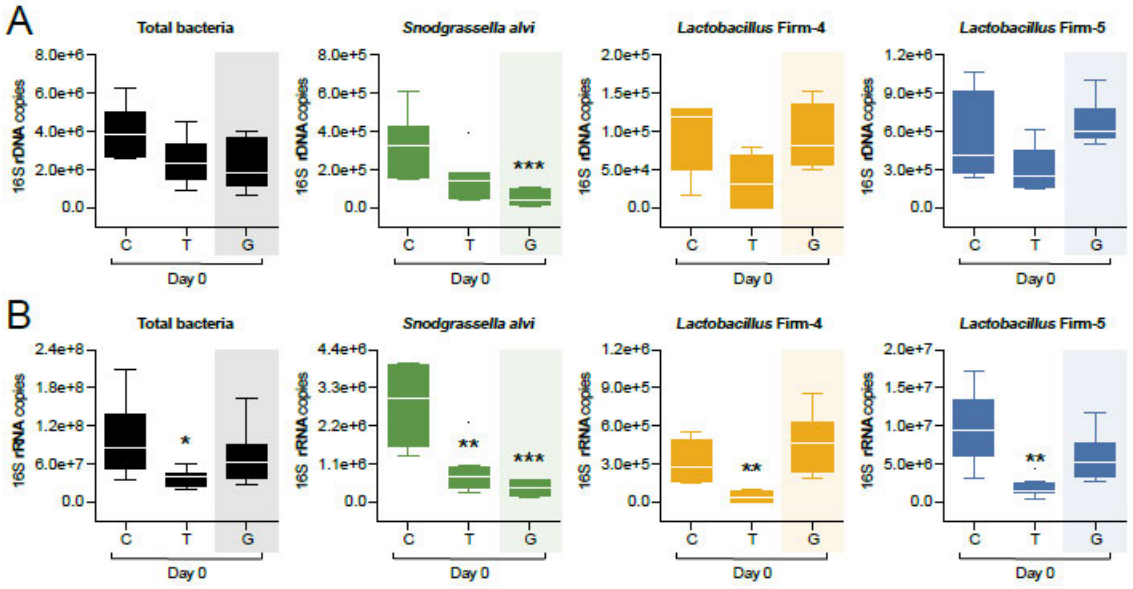
Taking into consideration the shortcomings listed above, the results of the hive experiment are not reliable and thus no conclusion can be drawn on glyphosate effect on gut microbiome size and absolute and relative abundances of the "core" species of the microbiome.

Colonization experiment

"Approximately 100" newly emerged workers (NEWs), which are supposedly nearly free of gut bacteria, were simultaneously exposed to an inoculum consisting of their normal microbial community and to glyphosate (5 µl of sugar syrup with 1 mM glyphosate ~1.7 µg glyphosate/bee; bees were exposed twice within 2 days). Of these, 15 bees were sampled in order to determine gut microbiome. In a second colonization experiment the exposure levels differed, i.e. the bees were exposed to 0.1 mM glyphosate during 5 days. Since no more information was available, it is not possible to calculate the total dose the bees consumed and to quantitatively compare the effects between the tests. In this case, only 8 bees per test group were sampled for DNA and RNA extraction.

In the first experiment, the average total bacterial abundance was slightly lower in glyphosate-treated bees, but this was not statistically significant. *S. alvi* was the most strongly affected member of the gut microbiota and its absolute and relative abundances were significantly lower in comparison with the control bees, while *Lactobacillus Firm-4* increased in relative abundance only. The authors concluded that glyphosate exposure during early development of the gut community can interfere with normal colonization by altering the abundance of beneficial bacterial species.

In the second experiment, the authors also analyzed changes in bacterial abundance after glyphosate exposure by extracting both DNA and RNA from the guts of treated and control bees. A positive control group was included, in which bees were exposed to tylosin, an antibiotic used in beekeeping. This antibiotic treatment was expected to perturb the microbiota, but the decrease was significant only for RNA samples. Glyphosate exposure resulted in non-significant decreases in total bacteria for both DNA and RNA assays. Effects of glyphosate treatment on absolute abundance were specific to *S. alvi*, which was the only assayed species showing significant reductions in absolute abundance, observed for both DNA and RNA assays. The authors concluded that significant effects were observed only on absolute abundance of *S. alvi* DNA and RNA.



Changes in gut microbiota composition following glyphosate exposure of age-controlled honey bees in the second colonization experiment. Boxplots of (A) 16S rDNA copies and (B) 16S rRNA copies of total bacteria, *Snodgrassella alvi*, *Lactobacillus Firm-4* and *Firm-5* for control (C), 0.1 mM tylosin treated (T), and 0.1 mM glyphosate-treated (G) bees at post-treatment Day 0 (n = 8 for each group).

Most of the shortcomings mentioned in the hive experiments are applicable here in the colonization experiment, e.g. (even smaller) sample size, unknown doses (per bee), lack of accounting for natural variations in gut microbiome among individuals. As a result, the colonization experiments are also considered unreliable.

Infection experiments

To determine whether glyphosate-induced perturbation of microbiota colonization affects host health, the susceptibility of glyphosate-treated bees to an opportunistic bacterial pathogen was measured in two experiments. NEWs were exposed to glyphosate during the stage of acquiring their normal microbial community. After 5d of treatment (first experiment: 0.1 mM glyphosate over 5 days; second experiment: 0.1 mM glyphosate over 5 d ~1.7 µg glyphosate/bee (assuming bees take on average 20 µl sucrose solution per day under captivity, the same likely applies for the first experiment, but it was not reported)), bees were challenged with *Serratia marcescens* kz19, an opportunistic pathogen commonly detected at very low frequencies in the bee gut. For bees lacking gut microbiota, *Serratia* challenge resulted in increased mortality relative to that observed for bees with a conventional gut microbiota, regardless of glyphosate exposure. For bees with a conventional gut microbiota, glyphosate treatment resulted in increased mortality after *Serratia* challenge. In bees exposed to glyphosate, but not challenged with *Serratia*, survival rates were not significantly affected by glyphosate and much higher (they were actually the highest from all tested groups) than in the *Serratia*-challenged groups, demonstrating that a direct effect of glyphosate on bees is not the basis of the high mortality of glyphosate-exposed, pathogen-challenged bees.

It was suggested by the authors, based upon the results above, that glyphosate reduces the protective effect of the gut microbiota against opportunistic pathogens and that *S. alvi* is the bacterial species most negatively affected by glyphosate exposure. The authors pointed out that *S. alvi* appears to give some immune protection, but not as fully as the whole gut microbiota.

The authors further hypothesize the reasons for the observed variation in sensitivity of certain strains of bacterial species toward glyphosate, which is outside of the scope of the present evaluation for ecological risk assessment. Only short conclusion on the topic is presented here: It was concluded that bee gut bacteria vary in glyphosate sensitivity at the species and strain levels and that *S. alvi* strains may vary in sensitivity to glyphosate in vivo. Thus, strain differences in glyphosate sensitivity may potentially contribute to the observed variation in the overall decrease in *S. alvi* abundance when bees with their native gut microbiota are exposed to glyphosate.

It it is not clear how the dose of the injected pathogen relates to potentially realistic doses (i.e. to what levels of *Serratia* the bees are naturally exposed). The authors state that the pathogen is naturally present in the gut at low frequencies, so injecting the pathogen does not seem to reflect natural situation. It is also not clear whether the possible observed effects were a consequence of the infection itself, or a consequence of infection and exposure together to glyphosate (as bees have a higher energy demand when exposed to two stressors), or a consequence of alterations of the honey bee immune response as a result of unaccounted for factors. Furthermore, the authors mention that the guts from 10 bees were pulled out, prepared, and were given to bees during 5 days until normal microflora was established. However, it is questionable what is the "normal" microflora, i.e. what is the baseline composition of the microflora. In addition, the bees were not fed with pollen (a source of proteins and important enzymes from nurse bees), which may influence the results as discussed above. Lastly, the doses were inferred by assuming that bees it 20 µl sucrose syrup per day, while in the EFSA GD on Bees (2013), the amount of the sugar bees consume is higher (32-128 and 34-50 mg/bee/day for

foragers and nurses, respectively), and therefore the exact exposure dose reported is considered incorrect or at least uncertain. Overall, the colonization/infection experiments are considered not sufficiently reliable.

Besides, although the study hypothesised that effects on the microbiome glyphosate may play a role in colony collapse, the positive control that was used for gut microbiome perturbation is an antibiotic commonly used in bee-keeping in some countries.

Overall conclusion
Several experiments were conducted in the study to show that exposure of adult bees might impact the gut microbiome leading to a greater susceptibility to pathogen infections.
On this aspect, the study is not directly relevant for the risk assessment but still relevant for the investigation of "other types of effects" not currently covered by the risk assessment scheme. It is considered by RMS as "additionnal data".

However, based on a number of shortcomings in each experiment (e.g. very small sample sizes, unknown rearing and experimental conditions, influence of hive, individual and age on gut microbiome, lack of confirmation of the levels of actual glyphosate exposure per bee, bees diets had no source of amino acids, injection of a pathogen at unjustified levels, etc.), the study is considered unreliable.

9. Non-target arthroprods

Data point:	CA 8.3
Report author	Leccia F. et al.
Report year	2016
Report title	Disruption of the chemical communication of the European agrobiont ground-dwelling spider *Pardosa agrestis* by pesticides.
Document Source	Journal of applied entomology (2016), Vol. 140, No. 8, pp. 609
Guidelines followed in study	none
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The aim of the study was to investigate the sexual chemical communication of the beneficial agrobiont spider *Pardosa agrestis* and its disruption by the glyphosate-based herbicide Roundup Klasik. A two-

choice olfactometer and Y-maze were used to study the effectiveness of female airborne and dragline pheromone cues and the disruptive effect of the pesticides.
The 3-h residues of Roundup Klasik significantly disrupted the male ability of *P. agrestis* males to follow female cues deposited on dragline silk.

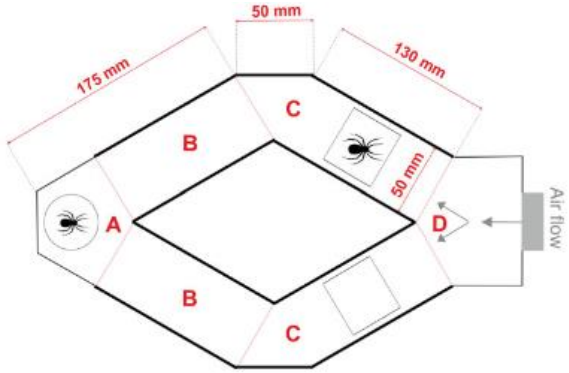
Materials and methods

Test item
Roundup Klasik (glyphosate isopropylamine salt (41.5%), detergent (15.5%) and water with other additives (43%).

Spider collection and housing
Juveniles of *Pardosa agrestis* were collected from cereal fields in Brno, Czech Republic, from April to June 2011 and in Prague, Suchdol, Czech Republic, in April 2014. The spiders were kept individually in translucent plastic tubes (15 mm diameter, 60 mm length) containing a layer of plaster of Paris at the bottom, which was moistened with a few drops of water at 3-day intervals to maintain humidity. The specimens were acclimatized in the laboratory at 21 ± 1°C and kept under a natural light/dark regime. They were fed with fruit flies (*Drosophila melanogaster*) and crickets (*Acheta domestica*) twice weekly. Spiders were fed a day before and between experimental trials to avoid unknown effects of starvation on spider response during experiments. The spiders were reared until they reached adulthood and kept for at least a further week before being used in experiments. All experiments were performed in an isolated and environmentally controlled room (no natural light, stable temperature and minimal disruption by visual stimuli).

Airborne cues: detection and response
One day prior to experimentation, males were tested to determine their readiness to mate. They were placed into a Petri dish (D = 15 cm, with filter paper at the bottom) with a female. Only males that followed the female or performed elements of courtship behaviour were used for experimentation. The effectiveness of the detection of female airborne pheromones by males was tested using a two-choice olfactometer (Fig. 1).

Figure 1. Two-choice olfactometer. Regions inside the olfactometer : entrance area (A), choice areas (B), treatment areas (C) and final section (D). The spider in the square container is a female; the tested male is in the cylindrical container.



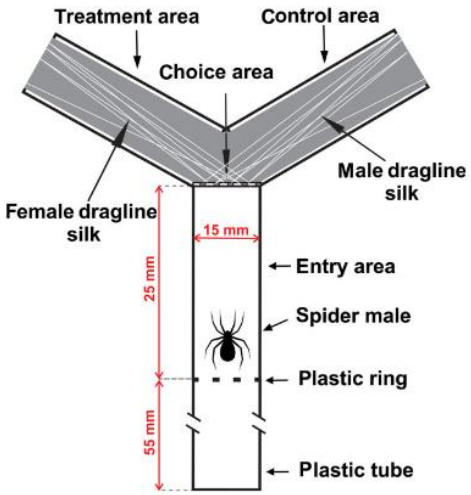
The olfactometer was constructed of glass with 5-cm walls covered in dark paper and an entrance bifurcated into two corridors. Airflow was generated at the end of both corridors with a miniature fan to push air through the apparatus. The airflow was regulated to be unidirectional. Four regions inside the olfactometer were defined: the entrance area (A), where the male was placed, the choice areas (B), which the male entered by selecting one of the two corridors of the olfactometer, two treatment areas (C), in the middle with a female in a container or an empty control container and the final section (D), an area beyond the treatment areas which was connected to a miniature fan with flow regulation.

A mature virgin female placed in cylinder container was inserted into the left or right corridor in one of the treatment areas (C). The specific corridor into which the female cage was placed was changed every 5 trials. Then, a sexually mature virgin male inserted into a cylindrical container. After 5 minutes of male acclimatization, the male was released from the container and the spider's behaviour was observed visually for the next 10 minutes or until the spider crossed into the final area (D). Males were allowed to choose between the corridor containing the female and the control (N = 30). During the experiment, corridor selection and whether the male made direct contact with the female was recorded (stopping at the female container and attempting to mate). The time taken for the male to reach the choice area, (B), was also recorded. The olfactometer was cleaned with ethanol and hot water, and dried completely between each trial.

Dragline cues: detection and response

The effectiveness of the detection of female dragline pheromones by males was investigated using a Y-maze. The Y-maze was comprised of three plastic corridors of dimensions 25 x 15 x 10 mm. The upper side was closed with a plastic lid. All corridors met in the choice area. The entrance area bifurcated into two corridors with different treatments (distilled water/pesticide) (Fig. 2).

Figure 2. 2Y-maze set-up



Dragline fibres were acquired from male and female adult virgins. Spiders were placed in a Petri dish of 150 mm diameter with a black paper base. Spider draglines were removed with tweezers after 60 min of spider activity. Ten to fifteen removed draglines were connected into lines spread longitudinally on black paper to be placed across the length of the treatment area. The choice area was connected to the intersecting treatment areas to test whether the male could detect female or male draglines as well as which draglines were followed significantly more. The side of the treatment area that received the pesticide treatment was changed every 5 trials. Mature virgin males (N = 30) were placed in a plastic acclimatization tube 55 mm in length, which fitted into the entrance of the Y-maze. After 1 min of acclimation in the tube, the tube was connected to the Y-maze.

Male behaviour was observed visually for the next 15 min or until the spider reached the end of a corridor. Three preference trials were performed as follows: (i) the preference between the corridor with female silk and the control corridor (no silk); (ii) the preference between the corridor with male silk and the control corridor (no silk); and (iii) the preference between the corridor with female silk and the corridor with male silk.

If a male remained in a corridor for more than 15 min, the authors considered that corridor as chosen by the spider. The experiment began when a spider crossed a line between the acclimatization tube and the entry tube of the Y-maze. The time of entry into the maze, the time of first contact with silk, the time at which a decision was made and the time when the spider reached the corridor exit were recorded and analysed.

There were no significant differences between the three preference trials (time of choice and time in chosen corridor). Between the testing of different males, the device was cleaned with ethanol. To test for the possible effect of side preference, the side treated with female silk was changed every three trials.

Disruption of chemical communication
The experimental design was the same as for the former preference trial involving the choice between the corridor with female silk and the corridor with male silk, except that the black paper installed in the treatment area was treated with pesticide residue (female dragline) or distilled water (male dragline). Silk from ten different males and females was installed on the paper floor of the Y-maze for each treatment. Thirty adult virgin males, randomly allocated numbers 1-30, were tested in groups of ten for their preference for dragline silk exposed to three treatments in the following order: treatment with distilled water (control experiment) and 3-h and 48-h residues of the glyphosate-based herbicide Roundup Klasik. Within each of the 3 treatments, each male (N = 10) was tested three times and never used twice in the same treatment. Altogether, 30 trials were performed for each treatment. Because of repeated use of the same male spiders, there was a 1-day recovery period after control trials and a 2- to 3-day recovery period after pesticide treatment. Each tested male spider (N = 33) was used one time per each treatment; this means that it was tested 4-5 times.

Application of Roundup
Roundup Klasik was diluted with distilled water according to the manufacturers' recommendations (15 mL of Roundup/L of distilled water) and applied onto the black paper floor (10 µL per 2.7 cm2 of paper) on the treated side. Distilled water was applied onto the black paper floor on the control side.

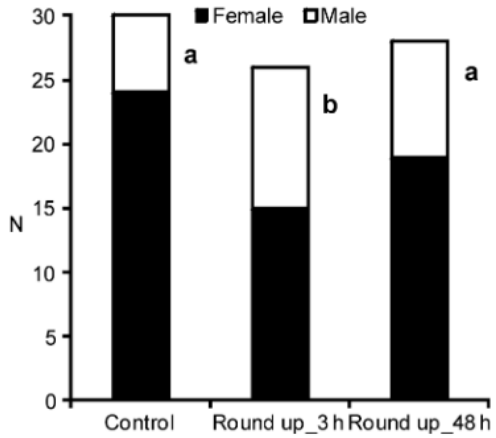
Statistics
Fisher's exact test was used to determine male choice with respect to airborne cues and cues deposited on silk. Differences in decision times between males selecting the female treatment side and males selecting the control side were tested and found to be statistically insignificant ($P > 0.05$). Generalized estimating equations with binomial error structure (GEE-b) were used to compare male choice assessed in a binary form in pesticide cues selection experiments. An 'exchangeable' correlation structure due to the small block size. This test did not reveal any correlation between male choice and its individual preference or previous decision. Posthoc comparisons among estimated parameters were made using treatment contrasts based on the Wald test, where all the pesticide treatments were compared separately with the control group. Statistical analyses were conducted within the R environment (R Development Core Team 2014). To avoid false positive results, Fisher's exact test was used to test male choice with respect to the effect of corridor side (left/right). There was no effect of corridor side on male choice ($P = 1$).

Results

Communication: airborne cues vs. cues deposited on silk
In the airborne pheromones experiments, males did not distinguish significantly between the corridor with the female and the control corridor (Fisher's exact test, $P = 0.797$, $n = 30$). In the dragline silk cues experiments, the males chose both the corridor with the female dragline (Fisher's exact test, $P < 0.001$, $n = 20$) and the corridor with the male dragline (Fisher's exact test, $P = 0.004$, $n = 20$) significantly more often than the control corridor (no silk). In the experiments in which male spiders chose between the corridor with the female dragline and the corridor with the male dragline, they chose the arm with the female dragline significantly more often (Fisher's exact test, $P < 0.001$, $n = 30$).

Cues disruption by the herbicide Roundup Klasik
Compared to the control (79%, n = 29), males were less likely to follow the female dragline exposed to the 3-h residue (54%, n = 26, GEE-b, $X2 = 3.88$, $P = 0.049$); thus the herbicide significantly affected male spider choice. Treatment with 48-h residues had no effect on male spider choice compared to the control (68%, n = 28, GEE-b, $X2 = 0.952$, $P = 0.33$) (Fig. 3).

Figure 3. Male choice with dragline silk treated with Roundup Klasik. Bars labelled with different letters denote significant differences at the $\alpha < 0.05$ level for 3-h residue.



Conclusion

Roundup Klasik significantly affected male spider choice, they were less likely to follow the female dragline exposed to the 3-h residue (P = 0.049) compared to the control.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Endpoints based on the impact of chemicals on spider pheromones are not used / required at EU level ecotoxicological regulatory risk assessments / glyphosate EU renewal.

Further points of clarification:
The formulation tested is not the representative formulation for the EU renewal of glyphosate (the representative formulation is MON 52276). Test substance purity and co-formulant identities are not stated. The authors acknowledged that some effects may have been caused by other components added to the formulations. As these are not stated, no conclusion on the formulation identity can be made.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The aim of the study was to investigate the sexual chemical communication of the beneficial agrobiont spider *Pardosa agrestis* and its disruption by the glyphosate-based herbicide Roundup Klasik. No sufficient information is available in the study report to demonstrate whether the tested formulation is comparable with the representative formulation.
The 3-h residues of Roundup Klasik (at recommended application rate) significantly disrupted the male ability of *P. agrestis* males to follow female cues deposited on dragline silk. Effect apparently transitory (no statistical difference with 48h old residues).
RMS notes the absence of clear conceptual link between sexual chemical communication and the specific protection goals for Non-target arthropods. It is agreed that it may play a role in the population health, but such link is not immediate in conceptual terms and not quantifiable. The results of this study are not relevant for the regulatory risk assessment.
RMs also notes that co-formulants are not stated.
This study is less relevant but supplementary (formulation). However the results presented are not relevant for the regulatory risk assessment given that no link between effct on sexual chemical communication parameters observed and effects on reproduction effectiveness was given. Reliability was not assessed.

Data point:	CA 8.3.2, CP 10.3.2
Report author	Lu Li-li et al.
Report year	2010
Report title	Effects of glyphosate on the growth and development of *Agasicles hygrophila*.
Document Source	Huanan Nongye Daxue Xuebao (2010), Vol. 31, pp. 22
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The experiment was designed to assess the effects of different concentrations of glyphosate on the development of *Agasicles hygrophila*. The treatment mass concentrations of glyphosate for eggs, Jarvae, pupae and adults were 0.41, 0.82, 1.23, 1.64 and 2.05 g/L, respectively. There was no difference in the hatching rate of eggs between the glyphosate treatment and the control group with the concentrations of glyphosate. However, the survival rates of larvae and adults treated by glyphosate were significantly lower than those in the control group. The survival rates of larvae and adults in the control group were 1.59- and 1.31-fold of that in the treated group with 2.05 g/L glyphosate, respectively.

Materials and Methods

Test organism
Alternanthera philoxeroides (alligator weed) and *Agasicles hygrophila* (alligator weed flea beetle) were collected from Chaohu City, Anhui Province. Cultured in the greenhouse of the Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, the experiment began after the *Alternanthera philoxeroides* had grown tender leaves.

Test item
41% glyphosate isopropylammonium saline, produced by Monsanto, USA.

Test conditions
The temperature is 26 °C and the relative humidity is 90% when illuminated; the temperature is 23 °C, the relative humidity is 90%, and the 12h/12h light is dark alternate when dark. An intelligent artificial climate box (PRX-4500), Ningbo Haishu Saifu Experimental Equipment Factory production, was used.

Effect of glyphosate on Agasicles hygrophila hatching rate
The monolayer filter paper is placed in the petri dishes (d =7.5 cm) with the same diameter, 1 mL of glyphosate wetting filter paper was added to each dish with a mass concentration of 0.41,0.82,1.23,1.64 and 2.05 g/L glyphosate solution respectively, and *Alternanthera philoxeroides* leaves or shoots with eggs (within 12 h) were added to each dish. The number of eggs per leaf was about 25~55, and then 2 mL of the corresponding concentration of glyphosate was added to the aforesaid petri dish to soak the eggs about 30 s. During the test, the filter paper was always kept moist to replace the clear water glyphosate as a control.

Effect of glyphosate on Agasicles larvae survival rate and age
The monolayer filter paper was placed in the same petri dish with the same diameter (d=12.5cm), each dish was treated with 2 ml of different mass concentration of glyphosate liquid wetting filter paper, and 15-30 the larvae of the newly hatched were added, and the larvae of the larvae were soaked with 3 ml corresponding concentration for 30 s, the excess liquid was sucked out, and the fresh *Alternanthera philoxeroides* was added. During the experiment, the filter paper was kept moist, the fresh water was replaced with glyphosate as the control, the fresh *Alternanthera philoxeroide* was replaced for 5 times, the culture dish was placed in the climate box, the larvae survival rate and the larvae were observed.

Influence of Glyphosate on 3rd instar Agasicles hygrophila larval pupation and emergence
The time of pupation could not be observed because the larvae of the third instar of *hygrophila* went into the stem to dissolve the pupae, so the time of pupation and the time of emergence were observed and calculated together in this experiment. The stems of the seeds were placed in the test tube (d=1.8 cm, l=18 cm), and 5 mL of different concentrations of glyphosate were added to each test tube, and then the larvae of the third instar were put into the test tube into the *Alternanthera philoxeroides* leaves. One larva in each tube, covered with gauze, water instead of glyphosate as control, treatment and control were repeated 10 times, every 12 hours to replace the test tube liquid, observe and record the time when the larvae drilled into the stem and Eclosion drilled out.

Effects on the Pre-oviposition period, eggs laying capacity and longevity of adult hygrophila
The fresh untreated *Alternanthera philoxeroides* tender shoots were placed in the bowl (d =12 cm, h =8 cm), and the different concentrations of glyphosate were added in the bowl, about 2 cm deep, and the adult with the emergence in Step 1.5 (within 12 hours of emergence) was inserted into the bowl, covered with gauze, treated with clean water as a control.10 replicates per treatment. The time and the number of spawning at the first time of the female *hygrophila* were observed and recorded every 12 hours. The tender shoots and water were changed every 24 hours until female and male *hygrophila* died. Record the time. If the male of each pair died first and the female did not, then the other males were added until the female died.

Effect of glyphosate on Agasicles hygrophila adults' survival rate:

Gastrotoxicity
Soak the *Alternanthera philoxeroides* for 30 s in advance with different concentrations of glyphosate, using water treatment as the control. After taking it out, it was put into a moisturizing petri dish (d =12.5 cm) with monolayer filter paper. 10 adults per dish, repeat it 5 times, replace the freshly pretreated *Alternanthera philoxeroides* every 24 hours and observe it for 14 days to record the death of *hygrophila*.

Contact
The monolayer filter paper was placed in a human petri dish (d=12.5 cm),10 adults were added to each dish, about 5 ml of different concentrations of glyphosate were added to soak the leaf armour for 30s, then the excess solution was sucked out with water treatment, and then the fresh hollow lotus seed grass was added. The filter paper was kept moist during each treatment 5 times, and the fresh hollow lotus seed grass was replaced every 24 hours and observed for 14 days.

Date Processing and Analysis
Using SPSS 13.0 and Excel 2003, variance analysis, multiple comparison and significant difference analysis were performed among different concentrations and controls.

Results

Effect of glyphosate on hatching rate and incubation period of Agasicles hygrophila
After different concentrations of glyphosate solution treated the *Agasicles hygrophila* eggs, each treatment and control group had a high hatching rate, and the hatching rate of the control group was 1.14 times that of the 2.05 g/l treatment group. ANOVA showed that the hatching rate of the eggs decreased with the increase of the concentration of glyphosate treatment, but there was no significant difference. The incubation period of the egg increased with the increase of the treatment concentration, and there was no significant difference between the mass concentration of 0.41, 0.82, 1.23 and 1.64 g/L and the control group, but the incubation period of the egg was significantly different with the control group when the mass concentration increased to 2.05 g/L (Table 1).

Table 1. Effects of different mass concentrations of glyphosate on the hatching rate of eggs of *Agasicles hygrophila*

P(glyphosate)/(g·L^{-1})	hatching rate/%	period/d
0	86.29 ± 3.25 a	5.9 ± 0.4 a
0.41	85.45 ±4.81 a	6.0 ± 0.4 a
0.82	83.14 ±3.36 a	6.5 ± 0.3 a
1.23	81.71 ±4.88 a	6.5 ± 0.4 a
1.64	79.58 ± 5.35 a	7.0 ± 0.3 a
2.05	75.53 ± 5.86 a	8.4 ± 0.4 b

1) Data in the table are average ±standard error (n=7).The difference was not significant for those with the same letter after the same column data (P<0.05)

Effect of glyphosate on larval growth and larval development
A. hygrophila
The survival rate of the *A. hygrophila* larvae decreased significantly with the increase of concentration after treatment with different concentrations of glyphosate. The survival rate of control larvae was the highest at 80.49%. The analysis of variance showed that when the concentration of glyphosate was 0.82, 1.23, 1.64 and 2.05 g/L, the survival rate was 63.31%, 56.58%, 55.34% and 50.65% respectively, which was significantly lower than that of control. Further analysis of variance showed that there was no

significant difference between the treatment group and the control group when the mass concentration was low, 0.41, 0.82 and 1.23 g/l, respectively. However, when the mass concentration increased to 1.64 and 2.05 g/L, compared with the low concentration treatment and the control group, the larval period was significantly prolonged and the difference was significant (Table 2).

Table 2. Effects of different mass concentrations of glyphosate on the period and the survival rate of larvae of *Agasicles hygrophila.*

P(glyphosate)/(g L^{-1})	larval survival rate/%	period/d
0	80.49 ± 2.40 a	11.0 ± 0.4 a
0.41	72.85 ± 6.68 ab	11.5 ± 0.4 a
0.82	63.31 ± 3.75 bc	11.5 ± 0.4 a
1.23	56.58 ± 6.69 c	11.9 ± 0.4 a
1.64	55.34 ± 5.83 c	13.0 ± 0.2 b
2.05	50.65 ± 4.91 c	13.0 ± 0.4 b

1) Data in the table are average ±standard error (SE) (n=5). The difference was not significant for those with the same letter after the same column data(P<005)

Effect of glyphosate on pupation and emergence of three-instar leaf A. hygrophila

Table 3 lists the time required for the larvae of the third stage of leaf armour to drill into glyphosate-treated *Alternanthera philoxeroides* grass stem internalization and pupae, and the control group required a longer period of 15.4 days. The analysis of variance showed that the larvae of the third age had internalized the pupae and the emergence period in different concentrations of glyphosate-treated *A. philoxeroides* grass stems significantly shortened compared with the control group.

Effect of glyphosate on pro-oviposition, oviposition and longevity of A. hygrophila

Glyphosate-treated *A. philoxeroides* with edible mass concentrations of 0.41, 0.82, 1.23, 1.64 and 2.05 g/L for adult *A. hygrophila*. The pre-oviposition averages were 8.4, 8.7, 9.0, 9.4 and 9.5 days, respectively, and the control group had a pre-oviposition average of 8.3 days. ANOVA showed that with the increase of glyphosate concentration, the early stage of pre-oviposition was prolonged, but the difference was not significant (Table 3).

The first oviposition of *A. hygrophila* treated with different concentrations of glyphosate was 39.6, 39.2, 38.6, 38.2 and 38.1, respectively, and the oviposition of *A. hygrophila* in the control group was 41.3. ANOVA showed that with the increase of glyphosate concentration, the oviposition decreased, but the difference was not significant (Table 3). The longevity of adult *A. hygrophila* was 29.3, 28.4, 27.5, 27.1 and 26.5 d, respectively, the control was 31.3 d. ANOVA showed that with the increase of glyphosate concentration, the life expectancy of *A. hygrophila* decreased. When the mass concentration was less than 0.82 g/L, there was no difference between the adult and the control group. However, the adult life was significantly shortened as compared with the control group when it was greater than 0.82g/L, and there was no significant difference in adult longevity between each concentration of glyphosate (Table 3).

Table 3. Effects of different mass concentrations glyphosate on the time of pupation and eclosion of larvae, pre-oviposition period of adult-adult longevity, the number of eggs of *A. hygrophila.*

P(glyphosate)/(g·L^{1})	3 instar larvae pupation and eclosion time/d	pre-oviposition/d	Adult longevity/d	Eggs/number
0	15. 4 ± 0. 5 a	8. 3 ±0. 3 a	31. 3 ± 1. 4 a	41. 3 ±2. 2 a
0.41	13. 1 ±0. 9 b	8. 4 ±0. 4 a	29. 3 ± 1. I ab	39.6 ± 2. 8 a
0.82	12. 7 ±0. 9 bc	8. 7 ±0. 3 ab	28. 4 ± 1. 2 ab	39. 2 ± J. 6 a
1.23	11. 3 ± 0. 8 bc	9. 0 ± 0. 4 ab	27. 5 ±0. 8 b	38.6 ± 2. 3 a
1.64	11. 1 ± 0. 9 bc	9. 4 ± 0. 4 ab	27. 1 ± 1. 3 b	38.2 ± 1. 4 a
2.05	10.4 ±0.5 c	9.5 ± 0.5 b	26.5 ± 1.4 b	38.1 ± 2.0 a

1) Data in the table are average ±standard error (n=10), The difference was not significant for those with the same letter after the same column data (P<0.05).

Effect of glyphosate on survival rate of adult A. hygrophila

The survival rate of *A. hygrophila* adults was high in both contact and gastrotoxicity treatment groups. The analysis of variance showed that with the increase of glyphosate concentration, the survival rate of *A. hygrophila* adults of the two treatments decreased gradually and was lower than that of the control group. The survival rates of adults with the two treatments in the control group were both high, 94.00% and 98.00% respectively. When the concentration of glyphosate was 0.41 and 0.82 g/l, there was no difference with the control group. When the mass concentration was 1.23 g/l, the survival rate of the adults with contact treatment was not different from that of the control group, and the survival rate of adults with gastrotoxicity treatment was significantly different from that of the control group. When the mass concentration was 1.64 and 2.05 g/L, the survival rate of the two treatments was significantly different from that of the control, and the survival rate of the gastrotoxicity treatment was higher than that of the contact treatment (Table 4).

Table 4. Effects of two treatments Oil the survival rates of adults of *A. hygrophila.*

P(glyphosate)/(g L^{-1})	survival rate/%	
	contact treatment	Gastrotoxicity
0	94. 00 ± 2.45 a	98. 00 ± 2 00 a
0.41	90. 00 ± 3.16 ab	92. 00 ± 2. 00 ab
0.82	88.00 ± 3.74 ab	88. 00 ± 3. 74 abc
1.23	82. 00 ± 2.00 abc	86. 00 ± 2. 45 bc
1. 64	78. 00 ± 6.63 bc	80. 00 ± 4. 47 c
2.05	72. 00 ± 5.83 c	74. 00 ± 4. 00 cd

1) Data in the table are average ±standard error (n=5). The difference was not significant for those with the same letter after the same column data (P<0.05).

Conclusion

Due to the serious problem of the prevention of recurrence of *Alternanthera philoxeroides* with herbicide, the control effect of its natural enemy hygrophila on the xerophytic type in low temperature area was also affected. The results of this study showed that glyphosate stimulated the pupation and emergence of the third-instar larvae of hygrophila. With the increase of glyphosate concentration, pupation and emergence of the *A. hygrophila* is become conspicuously shortened. Although glyphosate has a certain effect on adults, but even if the concentration is high, the survival rate is more than 70%, which can still maintain a certain population quantity. Glyphosate has a great effect on the survival rate of larvae *A. hygrophila*, and its survival rate is more than 50% when the concentration is high, which has a certain effect on the *A. hygrophila* population. In the experiment, the larvae feed on glyphosate at

high frequency. However, glyphosate will not be used so frequently in the field, so the concentration that *A. hygrophila* population can tolerate will be relatively high. It is recommended that spraying glyphosate during the comprehensive control should avoid the larval stage and the adult stage as much as possible. At the larval stage, if it should be applied, the mass concentration of glyphosate solution should be preferably less than 0.82 g/L. In the adult stage, the concentration should be less than 1.23 g/L.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The test substance is 41% glyphosate IPA salt. The study on *Agasicles hygrophila* was not conducted or based on a relevant NTA guideline.

Further points of clarification:
Alligator weed is an invasive species with a limited distribution in Europe. It is native to south America. In Europe, this species of aquatic plant has been added to the EU alien species list. The paper recommends the use of glyphosate to control alligator weed, but to avoid application during the larval stage of the weed flea beetle. This is a specific targeted application scenario for an invasive aquatic plant species. Aqautic uses are not on the proposed use table (GAP) for the representative formulation and therefore the data presented in this paper are not considered relevant to the glyphosate EU renewal.
In addition, the product used in the study is not sufficiently identified, but based on the stated '41% glyphosate IPA salt', this is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The experiment was designed to assess the effects of glyphosate (in a formulation not defined) on the development of Agasicles hygrophila (a weed flea beetle species). There was no difference in the hatching rate of eggs between the glyphosate treatment and the control group at all concentrations tested. However, the survival rates of larvae and adults treated by glyphosate were significantly lower than those in the control group. The survival rates of larvae and adults in the control group were 1.59- and 1.31-fold of that in the treated group with 2.05 g/L glyphosate, respectively.

This study was not conducted or based on a relevant NTA guideline.

RMS questions the relevance of the exposure. A monolayer filter paper was placed in the petri dish and was treated with glyphosate solutions wetting filter paper.
Results are all expressed in terms of concentrations but the volumes applied in Petri dishes are not explained.
Then the reliability of the effective exposures in this study is questionable.
The results are not relatable to EU regulatory risk assessment.

The test item is not well identified.

This study is relevant. However the results are not reliable.

Data point:	CA 8.3.2, CP 10.3.2
Report author	Rainio M. J. et al.
Report year	2019
Report title	Effects of a glyphosate-based herbicide on survival and oxidative status of a non-target herbivore, the Colorado potato beetle (*Leptinotarsa decemlineata*)
Document Source	Comparative biochemistry and physiology. Toxicology & pharmacology (2019), Vol. 215, pp. 47
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The direct toxic effects of the glyphosate-based herbicide Roundup® Bio, containing 360 g/L glyphosate, on survival of Colorado potato beetles (*Leptinotarsa decemlineata*), originating from Poland and USA, were investigated. Beetles originating from different continents from different populations were used to compare of the susceptibility to glyphosate exposure. Newly hatched larvae (3–6 days old) were tested at the concentrations of 100 and 1.5 % Roundup® Bio (360 and 5.4 g glyphosate isopropylamine salt/L). Larvae were treated to Roundup by pipetting a small drop (3 µL) on top of the larvae. Observations were made after 2, 24, 48, 72 and 96 hours.
Roundup® Bio at the high concentration (100 %, 360 g/L of glyphosate isopropylamine salt) significantly affected the survival of the Colorado potato beetle larvae. The test concentration at 1.5 % of Roundup® Bio had no major effect on survival. Roundup® Bio was dose-dependent and linked to exposure time.

Materials and methods

Test item
Roundup® Bio containing 360 g/L glyphosate (Monsanto, Missouri, USA).

Test organisms
The test organisms were Colorado potato beetles originated from Poland (Belchow) and USA (Vermont). The Vermont beetles were field-collected in 2010, after which it has been grown in the laboratory conditions in Jyväskylä, Finland. The beetles from Belchow were field-collected in 2010 and thereafter kept in laboratory conditions. Beetles from different continents were used to compare the susceptibility of beetles originated from different populations to glyphoate-exposure. Altogether, 1848 beetle larvae from 13 to 15 families (Belchow: 15 families, Vermont: 13 families) were used in the experiment. The newly hatched larvae were reared in petri dishes (with fresh potato leaves provided ad libitum) for 3–6 days.

Test design
Roundup® Bio treatment was conducted during the summer of 2014 in a licensed quarantine laboratory in the University of Jyväskylä. After rearing of larvae, the organisms were randomly divided into three groups: 1) high concentration (3 µl of 100 % Roundup Bio, 360 g/L of glyphosate isopropylamine salt), 2) low concentration (3 µL of 1.5 % Roundup Bio, 5.4 g/l of glyphosate isopropylamine salt) and 3) control group (3 µL of distilled water). The treatment groups were treated with Roundup (high and low concentration) by pipetting a small drop (3 µL) on top of the larvae, which were placed in a petri dish covered with filter paper. After the exposure, the larvae were kept without food for 2 h to ensure that they did not get the exposure via food. The control group was treated similarly but using water instead of Roundup. The larvae were exposed to Roundup and assessments were made after 2 h, 24 h, 48 h, 72 h and 96 h (with different larvae at each time point). Larvae from each treatment group were checked for mortality and immediately frozen at −80 °C for subsequent physiological analyses.

Statistics
All statistical analyses were performed with the SAS statistical software 9.4. (SAS, 2013). The survival of the larvae among the treatment groups (high dose, low dose and control), time points (2 h, 24 h, 48 h, 72 h and 96 h) and origins was analysed with a generalized linear mixed model (GLMM) with binary distribution and logit link function (events/trials syntax in GLIMMIX procedure). Family was used as a random factor to control for the non-independence of larvae used from the same family. Degrees of freedom were calculated with Kenward–Roger method and post-hoc pairwise comparisons were performed using Tukey's test. Differences in survival between the treatment groups in each time point separately were analysed likewise with GLMM (binary distribution and logit link function) separately for both populations using treatment as dependent variable and family as a random factor. Degrees of freedom and post- hoc tests were carried out as above. Differences in larval survival (using only control larvae) between the larvae of different origin in each time point were analysed likewise with GLMM (binary distribution and logit link function) with similar degrees of freedom method as mentioned above.

Results

Survival
Roundup® Bio treatment significantly affected survival of the Colorado potato beetle larvae (Table 1). Survival was lowest in the high concentration group (81.9 %), followed by the control group (92.8 %) and low concentration group (93.1 %). The low concentration and control groups did not differ from each other. The beetles originated from Belchow and Vermont populations did not differ from each other and there was no origin × treatment interaction, whereas time points had significant association to larval survival as also origin × time point interaction.

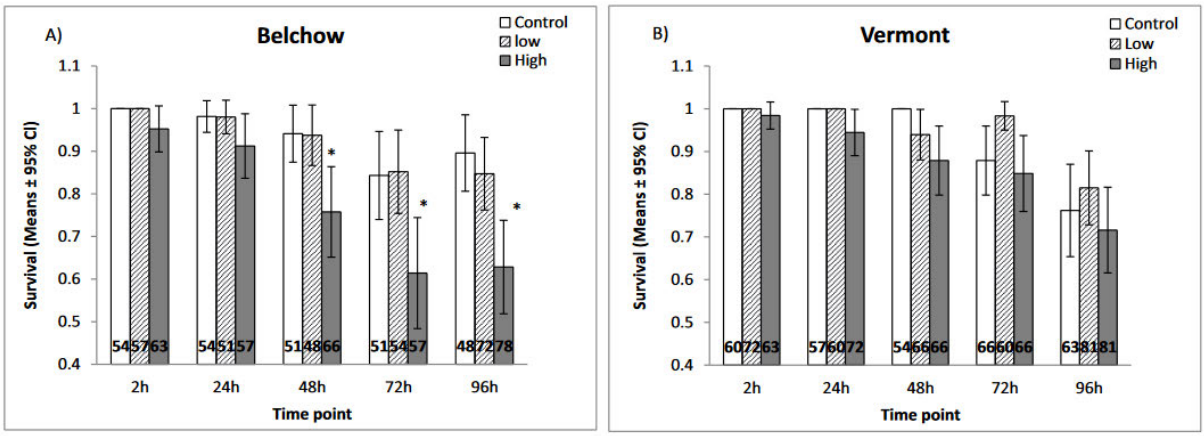
Table 1. The relationship between glyphosate treatments (high, low, control), origin (Belchow and Vermont) and time points (2 h, 24 h, 48 h, 72 h and 96 h) on survival of the Colorado potato beetle (Leptinotarsa decemlineata) larvae. Significant results are shown in bold.

	Survival	
Model[a]	F_{df}	p
Treatment	$26.06_{2, 1836}$	**< 0.0001**
Time point	$21.51_{4, 1836}$	**< 0.0001**
Origin	$3.70_{1, 53.97}$	0.060
Origin × time point	$2.53_{4, 1836}$	**0.039**
Origin × treatment	$2.48_{2, 1834}$	0.084

[a] GLMM with binary distribution and logit link function, family used as a random factor in the model.

Further analyses (separately for each time point) showed that larval survival between treatment groups differed also in each time point depending on time point and origin (Figure 1). In the larvae originating from the Belchow population, treatment groups differed at the 48 h ($F_{df} = 4.93_{2, 162}$, p = 0.008), 72 h ($F_{df} = 5.31_{2, 159}$, p = 0.006) and 96 h ($F_{df} = 7.86_{2, 195}$, p = 0.0005) time points, the high concentration group having significantly lower survival compared to the low concentration and the control group. However, at the 2 h ($F_{df} = 0.00_{2, 171}$, p = 0.999) and 24 h ($F_{df} = 1.76_{2, 159}$, p = 0.176) time points, no treatment differences were seen (p < 0.005). The larvae originating from the Vermont population, on the other hand, had no differences between the treatment groups in any of the studied time points (2 h: $F_{df} = 0.00_{2, 192}$, p = 0.999, 24 h: $F_{df} = 0.00_{2, 186}$, p = 0.999, 48 h: $F_{df} = 0.71_{2, 183}$, p = 0.494, 72 h: $F_{df} = 2.44_{2, 189}$, p = 0.090, 96 h: $F_{df} = 1.09_{2, 222}$, p = 0.339). When comparing the survival of the larvae of different origin in each time point by using only control larvae, only larvae at 96 h time point ($F_{df} = 4.32_{1, 109}$, p = 0.042) differed significantly between the larvae of different origin, but the other time point showed no differences (p > 0.05) in relation to larval origin.

Figure 1 (A + B). Survival of the Colorado potato beetle (*Leptinotarsa decemlineata*) larvae between the treatment groups in each time point separately in larvae of different origin (Belchow and Vermont). Bars represent survival (means ± 95% Cl) in each time point separately between treatment groups (white = control, striped = low, grey = high). Asterisks above the bars indicate significant differences between the groups in each time point (GLMM, p < 0.05).



Conclusion

Roundup® Bio at the high concentration of 100 % (360 g/L of glyphosate isopropylamine salt) significantly affected the survival of the Colorado potato beetle larvae. The test concentration at 1.5 % (5.4 g glyphosate isopropylamine salt/L) of Roundup® Bio had no effect on survival. Roundup® Bio was

dose-dependent and linked to exposure time.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The material and methods section lacks some important information. Newly hatched larvae from field collected beetles were used, however information on previous exposure to other chemicals or field history was not documented. Information on replicates, loading per replicate and test conditions were not reported. The preparation of the test solution was not specified. The test approach used does not follow a recognised test guideline and the rationale for the route of exposure and the dosing volumes used, is not described. The author indicates that a 100% Roundup Bio exposure in nature is unlikely to occur and that the high concentration mainly tests the physiological limits of the system including the antioxidant enzyme capacity of the beetles against the product. Exposure levels where significant effects were observed are unrealistic highlighting. There was no analytical verification, and the study was not performed according to GLP. Furthermore, endpoints based on biochemical analyses of larval homogenates cannot be applied in regulatory risk ecotoxicology assessment of non-target arthropods. Given the unrealistically high exposure levels used in the study, the non-guideline approach and the uncertainties as identified above the study is considered unreliable.

Assessment and conclusion by RMS:

The direct toxic effects of Roundup® Bio, containing 360 g/L glyphosate, on survival of Colorado potato beetles (Leptinotarsa decemlineata) were investigated. Beetles originating from different continents from different populations were used to compare of the susceptibility to glyphosate exposure. Newly hatched larvae (3–6 days old) were tested at the concentrations of 100 and 1.5 % Roundup® Bio (360 and 5.4 g glyphosate isopropylamine salt/L). Larvae were treated to Roundup by pipetting a small drop (3 µL) on top of the larvae. Observations were made after 2, 24, 48, 72 and 96 hours.
Only highest dose induced effects.
The dose rate tested is considered unrealistically high as mentioned by study author.
The study is not relevant (exposure unrealistic). Reliability was not assessed.

Data point:	CA 8.3
Report author	Saska P. et al.
Report year	2016
Report title	Treatment by glyphosate-based herbicide alters life history parameters of the rose-grain aphid *Metopolophium dirhodum.*
Document Source	Scientific reports (2016), Vol. 6, pp. 1-10
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant:	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B

See RMS analysis in RMS comment box	

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The authors investigated the sub-lethal effects of glyphosate on the aphid *Metopolophium dirhodum* (Walker), using an age-stage, two-sex life table approach. Three concentrations of the herbicide (low - 33.5, medium - 66.9 and high - 133.8 mmol dm^{-3} of active ingredient) and distilled water as the control were used. The LC_{50} of the IPA salt of glyphosate on *M. dirhodum* was equivalent to 174.9 mmol dm^{-3} of the active ingredient (CI95: 153.0, 199.0). The population parameters were significantly negatively affected by herbicide application, and this negative effect was progressive with the increasing concentration of the herbicide. A difference of two orders of magnitude existed in the predicted population development of *M. dirhodum* between the high concentration of the herbicide and the control. This is the first study that comprehensively documents such a negative effect on the population of an herbivorous insect.

Materials and methods

M. dirhodum (a laboratory strain that had been maintained for 15 years in the Crop Research Institute, Prague) was used as the model herbivore species. It is an oligophagous aphid species that occurs abundantly on leaves of all cereal crops in Central Europe in its summer phase, and is very easy to rear in laboratory cultures. Viviparous wingless or winged females are 2–3 mm long and develop through four larval instars. The aphids were maintained on young (stages 12–13 according to the BBCH scale) plants of winter wheat (*Triticum aestivum* Linnaeus) in a greenhouse with the temperature regulated to approximately 20 ± 1 °C and under a natural photoperiod.

Roundup Aktiv (Monsanto, Antwerp, Belgium) was used as the glyphosate-based herbicide in this study. This herbicide contains 229 g of the isopropylamine (IPA) salt of glyphosate (molecular weight = 228.18 g mol^{-1}) as the active ingredient (a.i.) per litre of the solution (1.004 mol dm^{-3}). The herbicide was applied on aphids by means a Potter Precision Laboratory Spray Tower with a spraying area of 110 cm^2.

Adult wingless aphids were placed on filter paper in a Petri dish (6 cm in diameter). For treatments, 2 ml of the solution (either distilled water or a solution of herbicide) was sprayed into the application chamber at a pressure of 3 bar.

An *a priori* toxicity test of the herbicide on *M. dirhodum* was made to detect the direct mortality within 24 h after the herbicide application. Eight different molar concentrations of the herbicide (diluted in distilled water) were used in this experiment: 16.7, 33.5, 66.9, 104.4, 133.8, 267.6, 501.8 and 1003.8 mmol dm^{-3} of the a.i. and pure distilled water as the control. These concentrations were chosen for the precise identification of the LC_{50} and LC_{90} of *M. dirhodum* after correction for the sprayed area and partial adhesion of the aerosol to the sides of the chamber and were equivalent to 40, 80, 160, 240, 320, 640, 1200 and 2400 ml of the commercial product diluted in 2 l of water applied per 100 m^2. Ten replicates were performed for each concentration, with each replicate containing 10 aphids. The mortality data were expressed as a binary vector containing the number of surviving and dead aphids per dish and treatment.

Three different molar concentrations of the herbicide (diluted in distilled water) were used in the life table study: 33.5 (low), 66.9 (medium) and 133.8 mmol dm^{-3} of the a.i. (high) and pure distilled water as the control. These concentrations were chosen according to the recommended dosages in agricultural systems by the manufacturer and were equivalent to 80, 160 and 320 ml of the commercial product diluted in 2 l of water applied per 100 m^2. The highest concentration used in this study represents the

maximum recommended concentration doubled, a situation that may occur in the field as a result of overlapping applications.

The sprayed aphids were immediately transferred individually to growing pots with 2–3 new plants of winter wheat (stage 11 according to the BBCH scale). Experiment 1 was conducted to study the long-term (i.e., over generations) sub-lethal effect of herbicide application on the life table of *M. dirhodum*, so the life table data were collected for the filial generation. We allowed the treated females to produce nymphs overnight and transferred the newly born nymphs (1–2 per female) to a new wheat plant (stages 10–11 according to the BBCH scale; 80–100 replications per treatment). Each day, the aphid instar was recorded, and after reaching adulthood, the newly born aphids from these adults were counted until death. The born nymphs were removed every day.

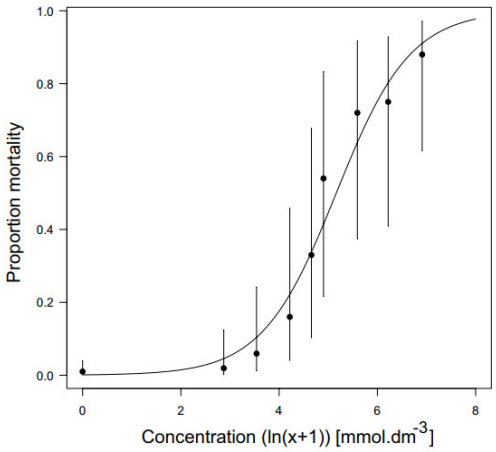
Experiment 2 was designed to study the short-term (i.e., in the same generation) sub-lethal effect of herbicide application on fecundity and survival of the treated *M. dirhodum*. A cohort of 23–28 newly hatched apterous females was treated with herbicide and monitored until death. Each day, the offspring were counted and removed. In both experiments, the individuals were transplanted to new wheat plants every three to four days to facilitate manipulation. The individuals included in this experiment were not mothers of the aphids included in Experiment 1.

The raw data of the individual insects (the survivorship, longevity, and female daily fecundity) were analysed according to the age-stage, two-sex life table theory using the computer program TWOSEX-MSChart. The growth of *M. dirhodum* population liberated from diseases, predation and parasitism was projected for each treatment based on the life table data using the program TIMING-MSChart and the approach described in Chi and Tuan *et al.*.

Results

The mortality of the aphids sprayed with the distilled water in the toxicity test was low after 24 h (2 individuals out of 400), suggesting that the method of spraying is not detrimental to the aphids. The mortality of *M. dirhodum* increased with the increasing concentration of herbicide (Fig. 1). Based on the results of the toxicity test of eight concentrations of the herbicide on *M. dirhodum*, the estimated values of the LC_{50} and LC_{90} were 174.9 mmol dm^{-3} of the a.i. (CI95: 153.0, 199.0) and 916.6 mmol dm^{-3} of the a.i. (CI95: 694.5, 1209.6), respectively. Based on this model, the mortality was estimated for the three concentrations of glyphosate-based herbicide used in the life table experiments: 10.4% (low), 22.2% (medium) and 33.8% (high concentration).

Figure 1. Observed proportion of *Metopolophium dirhodum* that died as a result of the application of glyphosate-based herbicide. The line represents the modelled response of the proportion mortality to increasing herbicide concentration based on the following logistic regression: logit (Proportion mortality) = −6.8515 + 1.3267 ln (Concentration + 1) [mmol dm^{-3} of the a.i.].



The development of filial *M. dirhodum* was rapid in all treatments and with negligible variation among the treatments in Experiment 1. The average duration of the adult stage varied across treatments and tended to decrease with herbicide concentration, which, together with the pre-adult survival that continuously decreased with herbicide concentration, caused that the average longevity to significantly decrease with each increase in the herbicide concentration. The reproduction parameters were also affected by herbicide treatments (Table 1); however, the lowest concentration of herbicide did not show any indication of negative effects, and the duration of the pre-oviposition period was even slightly shorter than for the control.

Table 1. Reproduction and life table parameters of Metopolophium dirhodum as affected by glyphosatebased herbicide application. Standard errors (SE) were estimated with bootstrapping (100,000 re-samplings).
The same letter within each row indicates that the groups of treatments are not significantly different from each other based on a paired bootstrap test. *significantly different between Experiments 1 and 2; NSnot significantly different between Experiments 1 and 2.

Parameter	Glyphosate treatment (Mean ± SE)							
	Control		Low conc.		Medium conc.		High conc.	
Experiment 1 – filial generation								
λ (d^{-1})	1.2793 ± 0.0052	a	1.2708 ± 0.0093	a	1.2217 ± 0.0140	b	1.1843 ± 0.019	b
r (d^{-1})	0.2463 ± 0.0041	a	0.2397 ± 0.0073	a	0.2002 ± 0.0115	b	0.1692 ± 0.016	b
R_0 (offspring individual^{-1})	41.15 ± 2.60	a	31.30 ± 2.80	b	17.13 ± 2.56	c	10.15 ± 2.03	d
T (days)	15.09 ± 0.25	a	14.37 ± 0.21	b	14.19 ± 0.27	b	13.71 ± 0.41	b
Fecundity (laid nymphs female^{-1})	41.16 ± 2.60	a*	38.17 ± 2.31	a*	29.29 ± 2.79	b*	20.09 ± 2.97	b*
Pre-oviposition period (days)	0.78 ± 0.09	ab	0.59 ± 0.09	a	0.87 ± 0.06	b	0.91 ± 0.11	b
Oviposition period (days)	13.91 ± 0.77	ab^{NS}	14.80 ± 0.76	a*	11.64 ± 1.05	bc*	9.82 ± 1.09	c^{NS}
Pre-adult survival	1.00 ± 0.00	a	0.82 ± 0.05	b	0.59 ± 0.07	c	0.35 ± 0.06	d
Female stage duration (days)	18.13 ± 1.24	a*	18.03 ± 1.18	a*	15.48 ± 1.20	ab*	13.78 ± 1.71	b*
Longevity (days)	27.06 ± 1.27	a	22.92 ± 1.44	b	16.26 ± 1.43	c	10.98 ± 1.19	d
Experiment 2 – treated generation								
Fecundity (laid nymphs female^{-1})	30.17 ± 3.52	a*	19.65 ± 3.88	b*	7.12 ± 2.76	c*	8.25 ± 2.09	c*
Oviposition period (days)	11.77 ± 1.07	a^{NS}	8.65 ± 1.54	ab*	4.27 ± 1.45	c*	7.15 ± 1.10	bc^{NS}
Female stage duration (days)	13.82 ± 1.44	a*	9.36 ± 1.81	a*	3.52 ± 1.05	b*	5.04 ± 0.97	b*

While each increase in herbicide concentration caused a significant decrease in *R0*, the *T* decreased regardless of the herbicide treatment compared to the control (Table 1). Both parameters of population

growth, *r* and λ, were unaffected by the lowest concentration and similarly decreased due to the medium and high concentrations of herbicide. The highest age-stage reproductive value (*vxj*) was estimated to occur at a similar age (9–11 days) in all treatments. The long-term sub-lethal effects of the herbicide application on the population biology thus occur prominently in the filial generation of the aphid *M. dirhodum*.

The fecundity, oviposition days and duration of adult stage were also estimated for the treated females in Experiment 2. As in Experiment 1, a negative effect of the herbicide treatment occurred on these population parameters. In the case of fecundity and female stage duration, the decrease in the observed values after herbicide application seemed to be more obvious in the parental generation (Experiment 2) than in the filial generation (Experiment 1). With the exception of oviposition days in the control and the high herbicide concentration, the values of all parameters were significantly lower in Experiment 2 compared to Experiment 1.

The population projection based on the age-stage, two-sex life table using the data from Experiment 1 shows that treatment with the glyphosate-based herbicide affects the population growth of *M. dirhodum*. According to the simulation, the difference in population size after 60 days will differ by two orders of magnitude and will reach approximately 8.76 million aphids with the control, 6.20 million at the low concentration, 0.60 million at the medium concentration and 0.09 million at the high concentration of the herbicide. The population growth curves (in a logarithmic scale) approach linearity after approximately 40 days, which suggests that aphid populations approached the stable age-stage distribution. The slopes of the regression lines that describe such linear population increase are equal to log(λ) for each cohort.

Conclusion

The main objective of this paper was to investigate the sub-lethal effects of a glyphosate-based herbicide on the population development of the cereal aphid *Metopolophium dirhodum*. Based on an age-stage, two-sex life table analysis, the acquired data provide evidence that this aphid species is sensitive to the doses (when applied by spraying) recommended by a producer of Roundup Aktiv, a glyphosate-based herbicide commercially available in the Czech Republic. All life table parameters of the filial generation, *r*, λ, *R0* and *T*, as well as the fecundity, duration of oviposition period, longevity and survival of both the treated and filial generations, were negatively and progressively affected by increasing the concentration of the glyphosate-based herbicide.

The life tables of *M. dirhodum* represent a typical aphid life table with high reproduction rate, short generation time, high intrinsic rate of increase and finite rate of increase of the population. Although a number of studies have estimated life tables of aphids using conventional methods, the age-stage, two-sex life table literature is sparse for aphids, and to the best of our knowledge, no life table data have been published for *M. dirhodum* so far. This is perhaps because it feeds exclusively on leaves and research attention focused mainly on aphids feeding on ears of cereals. Detection of the stage differentiation and stage structure in parthenogenetic populations is, however, also important. Data for other species of aphids have to be used for comparison, among which *S. avenae*, the species that feeds on ears of cereals, has been the most studied. In general, the life table parameters estimated in this study are somewhat similar to the published data on aphids, although the variation in the literature is huge for several reasons.

Importantly, the use of approaches other than life tables might deliver different results and conclusions on the effects of glyphosate-based herbicides. Using life tables, however, provides a more complete answer to the research questions related to the population biology of a species when the sub-lethal effects of pesticides are the focus. We therefore see a strong need for further studies on the sub-lethal effects of herbicides on non-target organisms through the use of age-stage, two-sex life tables. Biological and ecological interactions, even in simplified systems such as agricultural crops, are complex, and an

understanding of how pesticide applications modify the roles of the individual components of natural food webs including insect herbivores, such as aphids, would be useful.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The paper does not present endpoints that could be used in an EU level ecotoxicological regulatory risk assessment. A formulation was tested that is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276).

Further points of clarification: The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

A test of Roundup Aktiv (Monsanto, Antwerp, Belgium) on rose-grain aphid Metopolophium dirhodum was made to assess lethal and sublethal effects after the herbicide application.
The LC50 of the IPA salt of glyphosate on M. dirhodum was equivalent to 174.9 $mmol.dm^{-3}$ of the active ingredient (CI95: 153.0, 199.0). The population parameters were significantly negatively affected by herbicide application.
This LC50 cannot be directly used in the a standard risk assessment. RMS make an attempt to convert this LC50 in LR50 assuming 200 l of water applied per ha. This would result in LR50 of 418 g /ha. However this cannot be used as reliable endpoint. Besides the dosing of the aphids also depends of the volume of water applied on the petri dishes. So the endpoint is uncertain.
An other formulation (not the EU representative one) was used. Toxicity of the glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant system in the product.
Overall, the study is considered less relevant but supplementary and not reliable.

Data point:	CA 8.3
Report author	Tahir H. M. et al.
Report year	2019
Report title	Effect of Pesticides on Biological Control Potential of *Neoscona theisi* (Araneae: Araneidae)
Document Source	Journal of Insect Science (2019), Vol. 19, No. 2, pp. 1-6
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The present study was designed to record the effect of glyphosate on the mortality, avoidance behavior, foraging activity, and activity of acetylcholine esterase (AChE) and carboxylesterase (CarE) in *Neoscona theisi*. Glyphosate showed significantly higher mortality (30%) than control. Spider spent less time on glyphosate-treated surfaces. Glyphosate-treated *N. theisi* consumed less prey than untreated control spiders. Similarly, when *N. theisi* were offered glyphosate-treated prey, they consumed significantly less. Increased AChE and CarE activities were recorded in glyphosate-treated spiders compared to control group. Total protein contents were less in glyphosate-treated spiders than control group.

Materials and methods

Test item
Glyphosate (100 ml/100 liters per acre) was used in the experiment. The field-recommended doses were used as given in a hand book for agriculture extension agents on the pesticides registered with recommendations for safe handling and use in Pakistan.

Test organism
Live adult orb-web spiders were collected from agriculture fields of University of the Punjab Lahore. Sampling was conducted from April to August 2018. Adult spiders (only females) from fields were brought to the laboratory and placed individually in separate plastic jars (3 cm wide and 12 cm long). Mouths of jars were covered with mesh net cloth. Each jar also contained wet soil to maintain humidity at the bottom. Spiders were fed on house flies (*Musca domestica*) and acclimatized in the laboratory for 7 d before using them for experiment.

Susceptibility Tests
For conducting susceptibility tests against glyphosate, spiders were divided into two groups, i.e., 1) glyphosate group, and 2) control group. The number of spiders in each group was 10. Whatman filter papers were taken and dipped in the recommended field dose of glyphosate (1 ml/100 ml). Filter papers of control group were dipped in distilled water. Filter papers were allowed to air dry for 1 h at room temperature and then placed in petri plates. A single spider was released in each petri plate and allowed to expose to glyphosate, or water impregnated filter paper for 1 h. After exposure of 1 h, spiders were transferred to the clean jar. No food was provided to spiders during the experiment. The mortality was recorded after every 4 h till 24 h.

Avoidance Behavior
To investigate the avoidance behavior of spiders, round Whatman's filter papers were cut into two equal halves. One half of each filter paper was dipped in recommended field concentration of glyphosate (1 ml/100 ml) while other part of filter paper was dipped in distilled water. Filter papers were air dried for 1 h and then both parts were again joined with scotch tape and placed in petri plate. In each petri plate, single spider was released and time (in seconds) spent by each spider on glyphosate or distilled water treated part of filter paper was recorded. Spiders were allowed to acclimatize for 15 min before recording the data. For each spider, data were recorded for 30 min (1800 s). Fifteen spiders were used in this experiment. The experiment was replicated three times.

Foraging behavior
For this study, following two experiments were conducted.

Offering of prey to insecticide-exposed spiders
Adult spiders were divided into experimental and control groups. The number of spiders in each group was 10. Each spider was placed in a container (6 cm wide and 12 cm long). Leaves and twigs were placed in container as anchor points to build a web by spider. To standardized hunger level of spiders,

they were first fed with house flies at three different times in the day to the satiation level and then starved for 3 d. Each spider of experimental group was exposed for 30 min to the filter papers treated with sublethal dose of glyphosate (one-third of field dose), while each spider of control group was exposed to distilled water-treated filter paper. Spiders were exposed to glyphosate by releasing them on glyphosate impregnated filter paper in closed container. Spiders of both group were offered with same number of prey, *M. domestica* (n = 10). It was ensured that the size of flies should remain the same for each group. The number of flies consumed by spiders of experimental and control group were compared using independent *t*-test.

Offering of glyphosate-exposed prey to spiders

In this experiment glyphosate-exposed prey was offered to the spiders. *Musca domestica* were divided into two groups. Group I was exposed to glyphosate, group II was untreated. Similarly, spiders were divided into groups. Each group contained 10 spiders. Each spider of group I was offered prey (n = 10) that were treated with sublethal dose of glyphosate (one-third of field dose). The control group was fed on untreated prey. The numbers of prey consumed by spiders were recorded till 24 h. The number of flies consumed by spiders of experimental and control groups were compared using independent *t*-test.

Biochemical Tests

To measure activities of AChE and CarE in glyphosate-exposed and unexposed spiders, biochemical tests were performed in the laboratory. Activity of AChE was determined by Ellman et al. (1961) method. CarE activity was measured by Van Asperen (1962) method. Total protein contents were estimated by Bradford method (Bradford 1976).

Statistical analysis

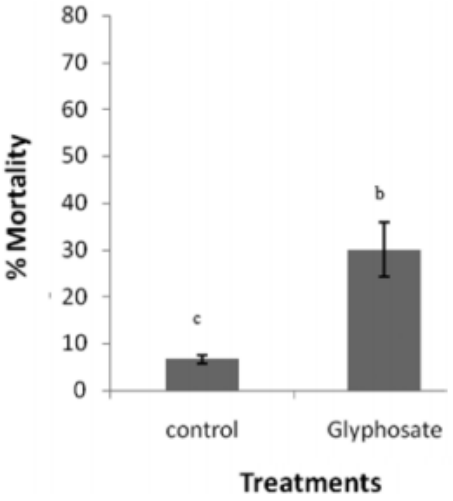
Normality of the data was assessed using Shapiro-Wilk test. One way analysis of variance (ANOVA) test was applied to compare the mortality among different treatments. The number of flies consumed by spiders of experimental and control group were compared using independent *t*-test. Paired *t*-test was used to compare the time spent by spiders on glyphosate-treated and control part of filter paper. The predation rate of spiders of control groups and glyphosate-treated groups were compared by using ANOVA followed by Tukey's test for multiple comparisons. Enzyme activity against glyphosate was compared by using ANOVA. All tests were performed using SPSS (version 22).

Results

Susceptibility Tests

Tukey's test showed that glyphosate-treated group showed significantly higher mortality than control (30%).Results of ANOVA showed statistically significant difference among treatments ($F_{3,46}$ = 16.00; $P < 0.05$).

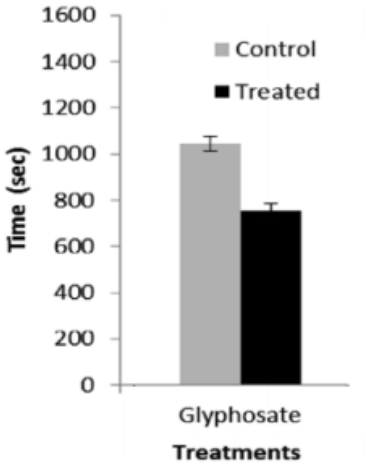
Figure 1: Response of *N. theisi* treated with the recommended field dose of glyphosate (1 ml/100 ml) in comparison with control group after 24 h post-treatment. Error bars are used to show the standard error.



Avoidance Behavior

The time (754 ± 31.2 s) spent by *N. theisi* on Glyphosate-treated part of filter paper was less than the time (1,044 ± 31.2 s) on untreated part of filter paper ($t = -8.48$; $P = 0.001$, Fig. 2).

Figure 2: Comparison of total time (seconds) spent by *N. theisi* on glyphosate- or water-treated filter paper. Error bars are used to show the standard error.

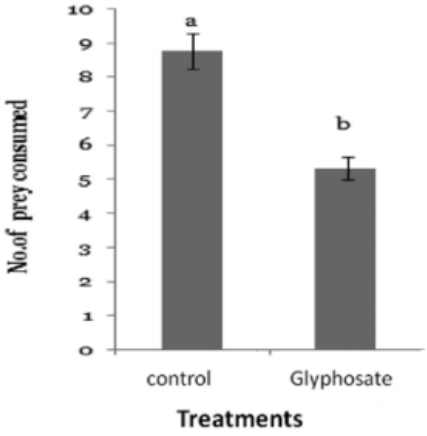


Foraging behavior

Offering of prey to insecticides exposed spiders

Result showed all spiders that were exposed with pesticides consumed significantly less prey than control group of spiders ($F_{3,46} = 14.34$; $P < 0.05$). The prey consumption of glyphosate-exposed spiders was 5.33 ± 0.33 (Fig. 3). Result of Tukey's test showed that prey consumption of glyphosate-treated spiders was significantly as compared control groups (Fig. 3).

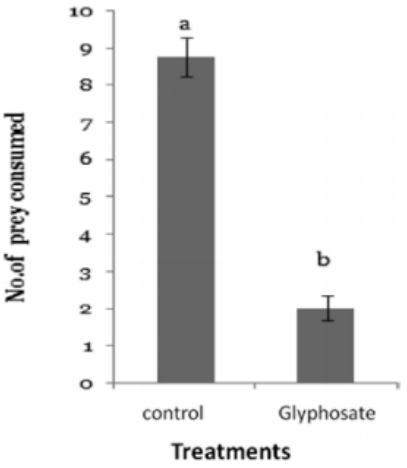
Figure 3: Feeding of *M. domestica* (*n* = 10) by *N. theisi* exposed to glyphosate (one-third of field dose) in comparison with control group (24 h). Error bars are used to show the standard error.



Offering of insecticides exposed prey to spiders

Overall prey consumption of control and the spiders which were offered pesticide-exposed prey is different ($F3,46 = 11.16$; $P < 0.05$). It is further evident from the Fig. 4 that prey consumption of all spiders that were offered glyphosate-treated prey differed nonsignificantly. The prey consumption was recorded as follows: control group (8.24 ± 0.72) > glyphosate-exposed prey (1.66 ± 0.12) (Fig. 4).

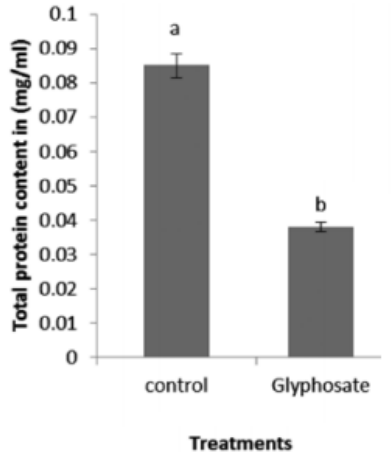
Figure 4: Feeding of *N. theisi* on *M. domestica* (*n* = 10) which were exposed to glyphosate (one-third of field dose) in comparison with control group (24 h). Error bars are used to show the standard error.



Estimation of total protein (mg/ml)

Total protein contents were decreased in glyphosate-treated groups as compared with control group. Significant difference was recorded in the total protein content of treated groups as compared with control ($F_{3,19} = 102.8$; $P = <0.001$). Results of Tukey's test showed that the glyphosate-treated group differ nonstatistically but differ from control (Fig. 5).

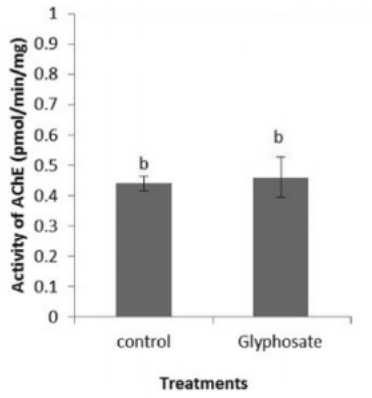
Figure 5: Total protein content of N. theisi treated with Glyphosate as compared to control group. Error bars are used to show the standard error.



Activity of AChE (pmol/min/mg)

There was significant difference in the level of AChE in glyphosate-treated group compared with control group ($F_{3,19}$ = 3.091; P = 0.047). It is evident from Fig. 6 that AChE activity of control- and glyphosate-treated group differ nonstatistically.

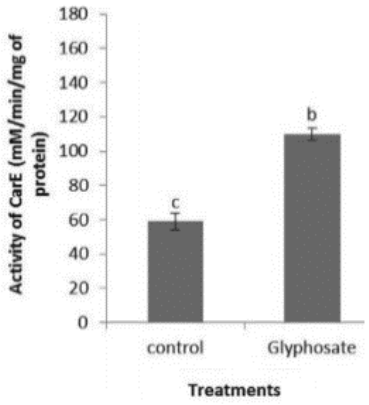
Figure 6: Activity of Acetylcholinesterases in *N. theisi* treated with glyphosate as compared to control group. Error bars are used to show the standard error.



Estimation of activity of CarE (mM/min/mg of protein)

There was significant difference in the level of CarE in glyphosate-treated and control group ($F_{3,19}$ = 14.41; P = <0.001). Results of Tukey's test showed that there is significant difference between control and treated group (Fig. 7).

Figure 7: Activity of carboxylesterases in *N. theisi* treated with glyphosate as compared to control group. Error bars are used to show the standard error.



Conclusion

The results of the study showed that selected spider species are susceptible to glyphosate. The observed mortality in *N. theisi* against field rate of glyphosate was 30%. A 50% reduction in prey consumption of *N. theisi*, which were exposed to sublethal dose of glyphosate (one-third of field rate) was observed. AChE, CarE, and protein contents were measured in spiders that survive after 24-h exposure of glyphosate. Glyphosate-treated spiders have increased level of enzymes as compared to untreated control group. Low level of protein contents was observed in the treated groups of spiders than control group.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Considered supplemental as the approach used does not follow an approach recognised at EU level for use in ecotoxicological regulatory risk assessment.

Further points of clarification:
The specification of the pesticide products used in the test was not stated beyond the active substance name. Therefore, the product relevance cannot be confirmed and the findings in this paper should be treated with high level of caution.

Assessment and conclusion by RMS:
Commercial product was used but the specifications of the pesticide products used in the test were not stated beyond the active substance name.
Glyphosate was used at the rate of 100 ml/100 liters per acre however density of the test material is unknown and no conversion in adequate dose rate is feasible.
Moreover, no biological data is available. This study does not follow standardized guideline.
The parameters investigated are relevant but the results are considered not reliable for regulatory risk assessment purpose.

10. Earthworms

Data point:	CA 8.4.1
Report author	Santadino M. *et al.*
Report year	2014

Report title	Glyphosate Sublethal Effects on the Population Dynamics of the Earthworm Eisenia fetida (Savigny, 1826)
Document Source	Water, air, and soil pollution (2014), Vol. 225: 2207, 8 pages
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The objective of this work was to determine the chronic, sublethal toxic effects of glyphosate in its commercial formulation Roundup® (Monsanto, SL at 48 %) on populations of *Eisenia fetida* and to evaluate the ecological importance of those effects on earthworms' demographic dynamics. Earthworm adults were randomly assigned to three glyphosate treatments: control (no glyphosate), regular dose for perennial weeds (6 L of formulated product/ha) and double dose (12 L of formulated product/ha). Six *E. fetida* individuals were placed in each pot. The test duration was 60 days. Two random pots were taken weekly from each treatment and the number of adults, individual weight, number of cocoons, and presence and number of young earthworms were recorded. A matrix analysis was performed with the data.

The matrix population model showed that glyphosate negatively affected the fertility of the *E. fetida* eggs and, consequentially, impacts the long-term dynamics of its populations. The viability population analysis indicated that in the absence of the disturbance, earthworm populations would increase in size. Furthermore, the matrix population model showed that while the control population had a positive growth rate, both glyphosate treatments showed negative growth rates. The sensitivity analysis shows that the most important parameters in the population dynamics were adult survival and fecundity for the control. When exposed to glyphosate, the most sensitive parameter in the population dynamics was the survival of the young turning into adults.

Materials and Methods

Test item
Glyphosate herbicide in its commercial formulation Roundup® (Monsanto, SL, 48 %).

Experimental Design
The test was carried out in pots under controlled temperature (25 ± 3 °C), humidity (80 %), and light: dark 12 h:12 h conditions. Twenty-four pots were arranged in three treatments. Each pot was filled halfway up with sifted soil from the upper 10 cm of an Argiudol soil (USDA 2010). On the surface of each pot, a layer of finely chopped plant material was added to add fresh organic food for the earthworms. In each pot, six weighed adult organisms of *E. fetida* were placed.
After 6 days of acclimation, glyphosate herbicide was added in its commercial presentation Roundup® with the equivalent dose for perennial weeds (6 L of formulated product/ha) and also a double dose

(12 L of formulated product/ha) as follows: (a) Control (treatment 0): 50 cm^3 of distilled water; (b) dosage 1 (treatment 1): 0.024 cm^3 + distilled water to complete 50 cm^3; (c) dosage 2 (treatment 2): 0.048 cm^3 + distilled water to complete 50 cm^3. For each treatment, eight replicates were made. Every 10 days, fresh food was added to each pot.
At days 12, 21, 28, and 40 after acclimation, two pots were randomly chosen from each treatment. The samples were manually examined, and the number of adult earthworms, their individual weights, the number of cocoons, and presence and number of young earthworms were recorded. Before discarding the samples, the cocoons were separated and incubated in Petri dishes over humid filter paper, and egg fertility was registered as well.
With these data, the following demographic parameters were calculated: survival of adults, fecundity, and cocoon's fertility. Young individuals were found in several pots probably due to cocoons which hatched before each sampling. However, the initial number of youngsters was unknown, and as a consequence to build the matrices, young survival was assumed to be equal to that of the adults (S_{23}=S_{33}).

Statistical Analyses
The number of earthworms per pot, the total weight per pot, the mean individual weight per pot, the fertility per pot and the number of cocoons per pot, and cocoon fertility were analyzed with ANOVA (α=0.05).

Population Dynamic Matrix Model
A matrix population model (MPM) of the earthworms' dynamic was build. For the tested species, the collected data correspond to several stadia on the life cycle of the earthworms, thus allowing testing the long-term sub-lethal effects on its population dynamics.
The matrix population model is useful to test for disturbance effects on the population dynamics, taking into account different survival, fecundity (*f*), and fertility (S_{21}) parameters. A Leslie matrix was used with a structure for a population of three stages. The Leslie matrix assumes that the chosen time lapse is such that the organisms that do not go on to the next stage die and that all of the adults die as well.
As the survival of adults was considered within the period, therefore, there is a diagonal term S_{33} that is not null. However, the other terms of the diagonal cannot be ignored, and it is known that only the adult individuals reproduce.
In these matrixes, the eigenvalue of greater module (called λ_1) gives an indication of the asymptotic dynamic of the population and the logarithm of its module is the intrinsic growth rate of the population (*r*). This means that the population grows if the module of λ_1 is greater than 1 (or $r>0$). The data obtained allows the estimation of different elements of the B matrix for *E. fetida*, under different glyphosate doses.
A sensitivity analysis was performed in the matrix built. In a population matrix, the eigenvalue of higher module determines the asymptotic behavior of the population.

Results

Glyphosate Effects on E. fetida Number and Biomass
No significant differences were determined for total weight per treatment or for the number of individuals when considering time as co-variable. The number of cocoons accumulated per treatment increased significantly ($p<0.05$) with the glyphosate dose. The fertility of these cocoons was significantly lower in the treatment with the higher dose than in the other treatments.

Effects of Glyphosate on the Population Dynamics of E. fetida
The demographic matrices obtained using the population parameters are presented in the following:

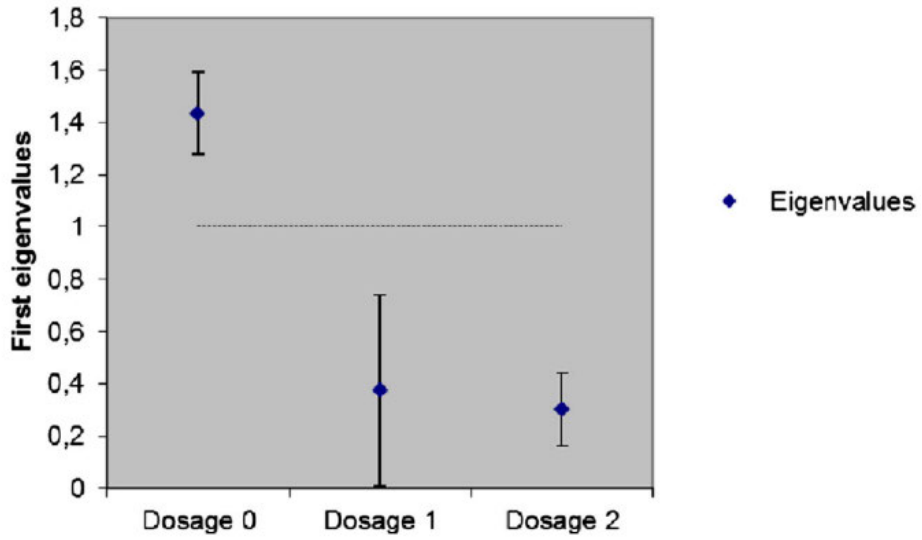
$$\text{Dosage 0: } A_1 = \begin{bmatrix} 0 & 0 & 0.04 \\ 1 & 0 & 0 \\ 0 & 1 & 1 \end{bmatrix}$$

$$\text{Dosage 1: } A_2 = \begin{bmatrix} 0 & 0 & 1.7 \\ 0.76 & 0 & 0 \\ 0 & 0.07 & 0.07 \end{bmatrix}$$

$$\text{Dosage 2: } A_3 = \begin{bmatrix} 0 & 0 & 0.81 \\ 0.84 & 0 & 0 \\ 0 & 0.04 & 0.04 \end{bmatrix}$$

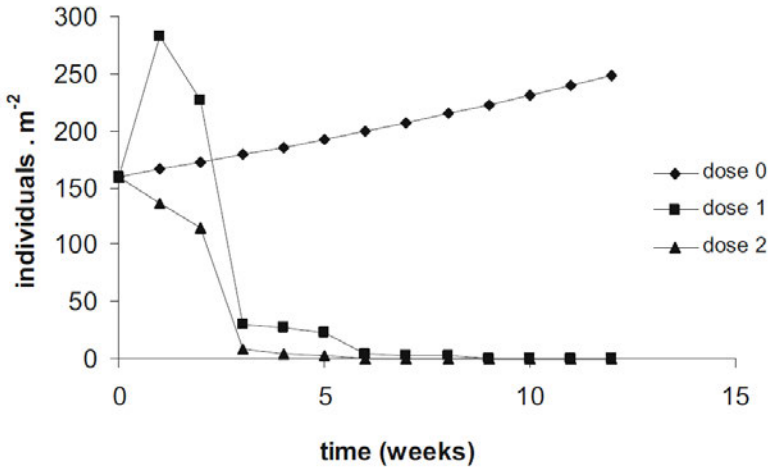
The larger than 1 eigenvalue in the case of the controls indicated that the population is growing, while the lower than 1 eigenvalues for the other treatments showed that the population size diminished in the long run (Figure 1).

Figure 1. Matrix population model eigenvalues for the three treatments. Mean ± SE shown. Values below the survivorship threshold (dotted line) indicate population diminishing in the long run, that could lead to local extinction.



The long-term projection indicated that populations under the chronic effect of glyphosate presented a negative growth rate, which could lead to local extinction. The lowest dose showed an initial increase in growth rate after the application of the pesticide. This result was likely caused by an increase in the fecundity as an initial response to stress. However, the final result showed both treatment populations having a negative growth rate (Figure 2).

Figure 2. Population dynamics projections according to the results of the matrix population model. Results show population having a positive growth rate for the controls and an overall negative growth rate for both glyphosate treatments. Both treatment populations are predicted to go locally extinct after 6 weeks.



In the treatments with glyphosate, the number of cocoons per adult was higher than those of the controls but the survival of juveniles and adults and the fertility were lower than in the control treatment. A sensitivity analysis of the matrices built (Table 1) shows that in the controls, the most sensitive parameter (the one that modifies the eigenvalue of the matrix the most) is the survival of the adults, while in the treatments with glyphosate, the most sensitive one is the survival from juveniles to adults.

Table 1. Sensitivities of the population parameters for each treatment. Adult survival is the most sensitive parameter for the controls, while survival of young turning into adults is the most sensitive parameter explaining the dynamics for both glyphosate treatments.

Parameter	Dosage 0	Dosage 1	Dosage 2
f (individual fecundity)	0.87	0.09	0.12
S_{21} *(egg fertility)*	0.03	0.20	0.12
S_{32} *(survival of young turning into adults)*	0.03	2.13	2.50
S_{33} *(adult survival)*	0.93	0.37	0.36

Conclusion

The results presented above showed that the biomass, the number of earthworms, and the dynamics of the earthworm populations could be affected by the regular use of herbicides in agriculture. The matrix population model showed that glyphosate negatively affected the fertility of the *E. fetida* eggs and, consequentially, impacts the long-term dynamics of its populations. The viability population analysis indicated that in the absence of the disturbance, earthworm populations would increase in size.

The matrix population model was used to calculate the asymptotic rate of population increase, showing it to be negative for high glyphosate levels. The sensitivity analysis shows that the most important parameters in the population dynamics were adult survival and fecundity for the control. When exposed to glyphosate, the most sensitive parameter in the population dynamics was the survival of the young turning into adults.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The chronic laboratory study with *E. fetida* was not performed according to a recommended guideline and thus, no validity criteria were given. Insufficient information is provided on the experimental design, as no information on the soil characteristics and the application of the test item is given. Only two test item treatment rates, without giving any rationale for choosing the higher dose, and a negative control were tested, but no positive control. No information on underlying raw data is given, i.e. number of control mortality, number of juveniles and cocoons etc. Finally, there are no quantifiable endpoints presented in the paper, considered applicable to an EU level ecotoxicological regulatory risk assessment.

Further point of clarification
The formulation used is not sufficiently identified to exclude the presence of POEA surfactant in the product. As co-formulants were not identified in this paper, the uncertainty associated with whether the product contained POEA or not, suggests that the findings in this paper should be treated with high level of caution. In addition, regulatory studies conducted with earthworms exposed to glyphosate containing products has demonstrated a lack of acute and chronic effects on earthworms exposed at equivalent test rates.

The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The objective of this study was to determine the chronic, sublethal toxic effects of glyphosate in its commercial presentation as Roundup® (Monsanto, SL at 48 %) on populations of E. fetida and to evaluate the ecological importance of those effects on earthworms' demographic dynamics.
The description of the formulation is not detailed and no information is provided on the surfactants of the formulation used. The study is less relevant but supplementary.
Moreover the experimental design is not sufficiently described to assess its reliability. Endpoints that could be used for risk assessment are not reported. RMS considers this study not reliable and cannot be taken into account for the assessment of the active substance glyphosate itself.

The study is considered less relevant but supplementary and not reliable.

Data point:	CA 8.4.1
Report author	Stellin F. *et al.*
Report year	2018
Report title	Effects of different concentrations of glyphosate (Roundup 360®) on earthworms (*Octodrilus complanatus, Lumbricus terrestris* and *Aporrectodea caliginosa*) in vineyards in the North-East of Italy
Document No	Applied Soil Ecology (2018), Vol. 123, pp 802

Guidelines followed in study	None
Deviations from current test guideline	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities (literature publication)
Acceptability/Reliability: as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study has not been conducted according to a recognized test guideline and there are no validity criteria presented. There is no information on the choice of test duration and the experimental design is not sufficiently described. A formulation was tested, but this is not the representative formulation for the glyphosate EU renewal (the representative formulation is MON 52276). No information is given on the set-up of the spray solution, how application was carried out and at which volume. For the soil sampling, the time point of sampling is not stated and no information on storage conditions of the soil prior to use in the study is given. Additionally, information on the soil depth in the experimental test containers is not mentioned. Similarly no information on food and environmental conditions during the exposure period (e.g. temperature, soil moisture, light conditions) are available. Finally, there are no quantifiable endpoints presented in the paper which could be used in the ecotoxicological regulatory risk assessment.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

The co-formulants are not stated but the study author noted that Roundup360® was made with a polyethoxylated tallow amine surfactant that is more toxic than glyphosate itself.
POEA is not authorized in plant protection products containing glyphosate (European Commission, August 2016). Some effects were observed but it is not possible to discriminate between glyphosate and POEA.
Due to presence of POEA, the study is considered not relevant by RMS. Reliability was not assessed.

Data point:	CA 9
Report author	Gaupp-Berghausen M. *et al.*
Report year	2015
Report title	Glyphosate-based herbicides reduce the activity and reproduction of earthworms and lead to increased soil nutrient concentrations.
Document No	Scientific reports (2015), Vol. 5, pp. 12886

Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The knowledge of potential side-effects on non-target soil organisms, even on such eminent ones as earthworms, was stated as very scarce. In a greenhouse experiment, the authors assessed the impact of the most widely used glyphosate-based herbicide Roundup on two earthworm species with different feeding strategies. The researchers hypothesized that herbicide application would stimulate earthworm activity and reproduction due to the increased availability of dead plant material that earthworms can use as food source. As a knock-on effect, they expected that herbicide application via its effects on earthworms would also alter water infiltration, soil nutrient availability, and decomposition. To test their hypotheses, the researchers established weed communities comprising of a grass, a leguminous herb and a non-leguminous herb species commonly occurring in arable agroecosystems or garden beds. To these weed communities they added vertically burrowing or horizontally burrowing earthworm species. Eight weeks after planting, the vegetation in half of the mesocosms was treated with a lower-than-recommended dose of glyphosate-based herbicide. The authors contend that the surface casting activity of vertically burrowing earthworms (*Lumbricus terrestris*) almost ceased three weeks after herbicide application, while the activity of soil dwelling earthworms (*Aporrectodea caliginosa*) was not affected. Reproduction of the soil dwellers was reduced by 56% within three months after herbicide application. Herbicide application led to increased soil concentrations of nitrate by 1592% and phosphate by 127%, pointing to potential risks for nutrient leaching into streams, lakes, or groundwater aquifers. These herbicide-induced impacts on agroecosystems were considered particularly worrisome to the authors because these herbicides have been globally used for decades.

Materials and methods

The experiment was performed between March and July 2013 in a greenhouse at the University of Natural Resources and Life Sciences Vienna (BOKU), Austria. Thirty six plastic pots (volume: 45 l, diameter: 42 cm, depth: 39 cm) filled with a 70:30 (vol/vol) mixture of soil from an arable field (soil type: Haplic Chernozem; BOKU Experimental Farm Gros Enzersdorf) and quartz sand (grain size 1.4–2.2 mm) were used to create mesocosms.

The mesocosms were planted with three types of plant species: the grass *Dactylis glomerata* L., the leguminous herb *Trifolium repens* L., and the non-leguminous herb *Taraxacum officinale* F.H.Wigg. The three species are common weeds on agricultural fields (e.g. arable land, vineyards) across Central Europe. Plants were germinated from seeds obtained from a commercial supplier specialized in wild plant populations (Rieger-Hofmann GmbH, Blaufelden-Rabholdhausen, Germany). When seedlings were 1 cm high, 17 seedlings per species were transplanted to the pots in a triangular pattern (5.5 cm

between plant individuals; plant density: 51 plants mesocosms^{-1}). During the experimental period, each mesocosm was irrigated equally using an automatic sprinkler system; mesocosms were placed on slats, allowing for free drainage.

Three weeks after planting, three earthworm treatments (n = 12) were established. The thirty-six mesocosms either received five specimens of adult vertically burrowing (anecic) *Lumbricus terrestris* L. (Lt) mesocosm^{-1} (25.5; 0.7 g mesocosm^{-1}; ~183 g m^{-2}), ten adult/sub-adult specimens of the horizontally burrowing (endogeic) *Aporrectodea caliginosa* Savigny (Ac) mesocosm^{-1} (12.09; 0.30 g mesocosm^{-1}; ~87 g m^{-2}), or no earthworms (NoEw).

Eight weeks after planting, mature plants (*D. glomerata* was about 40 cm high, *T. repens* 19 cm, *T. officinale* 31 cm) of half of the mesocosms were treated with the herbicide 'Roundup®' (treatment + H), whereas the other half of the mesocosms remained untreated (treatment –H). Each + H mesocosm was sprayed with 7.2 ml of 'Roundup® Alphee' (glyphosate concentration 7.2 g/L; Scotts Celaflor, Mainz, Germany) on two consecutive days (in sum 14.4 ml), and 10 ml of 'Roundup® Speed' (glyphosate concentration 7.2 g/L; Scotts Celaflor, Mainz, Germany) two days afterwards. In total for all applications, 176.12 ml m^{-2} of herbicide was applied which is 53% lower than the recommended plant-based application rate of 1000 plants l^{-1} for 'Roundup® Speed' and 62% lower than the recommended dose of 800 plants l^{-1} for 'Roundup® Alphee'.

Results

Herbicide application initially stimulated surface casting activity of *L. terrestris*, however the number of produced casts ceased dramatically about one week after herbicide application; in contrast the surface casting activity of this species remained nearly constant when no herbicide was applied.
Not only did exposure to herbicide reduce the number of surface casts produced, it also reduced the mean mass of individual casts (546; 202 mg cast^{-1} vs. 1,408; 140 mg cast^{-1}).

Compared to non-herbicide treated mesocosms, cumulative cast mass produced by *L. terrestris* four weeks after herbicide application was reduced by 46% compared to untreated mesocosms (560 g m^{-2} vs. 1,032 g m^{-2}; $P < 0.001$). Surface casting activity and cast mass production of the soil-dwelling earthworm species, *A. caliginosa*, was not affected by herbicide application. Although the studied earthworm species differ in their feeding behavior, both have been shown to cast on the soil surface when foraging for leaf litter and other organic material. The peak in surface casting activity observed after herbicide application was therefore likely the consequence of an increased availability of dead leaf material.

Figure 1. Activity of vertically burrowing earthworms before and after the herbicide application (-H, without herbicide; +H, with herbicide). (A) Daily surface cast production, (B) cumulative cast production over the course of the experiment, (C) time course of soil temperature (temp) and soil moisture (moist) (N = 6, mean SE). Red band marks period of herbicide application. P-values from two-sample Wilcoxon tests performed for the pre- and post-herbicide periods.

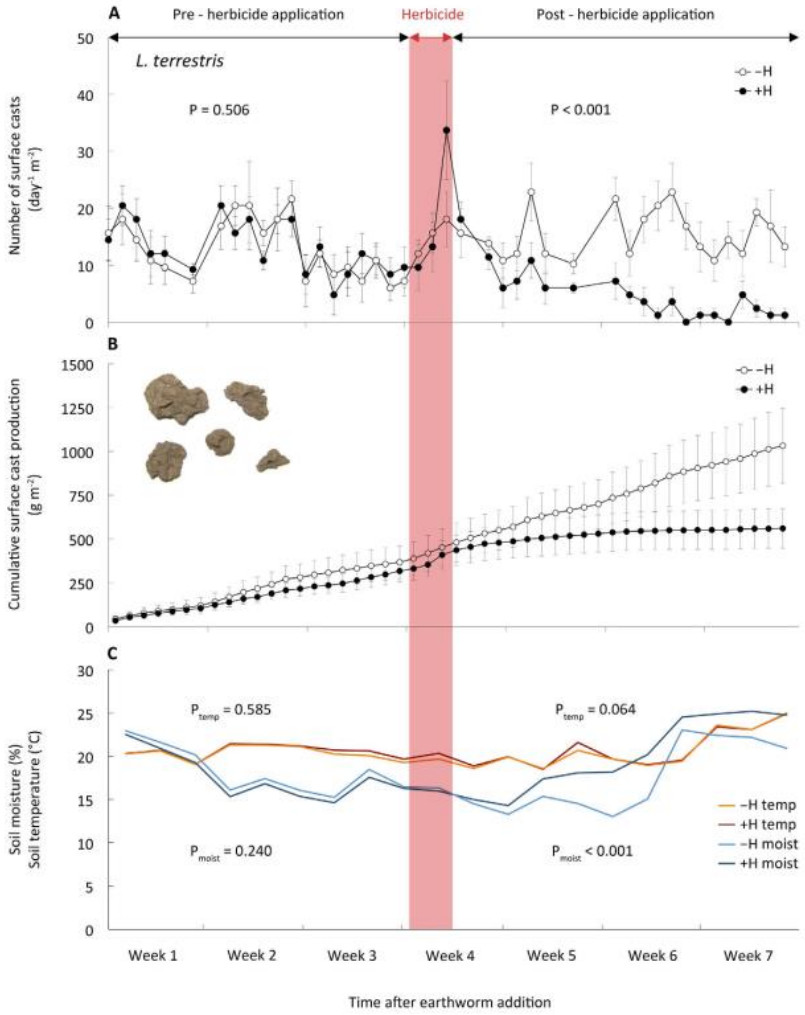


Figure 2. Percentage of cocoons with hatchlings of a vertically burrowing (*L. terrestris*, Lt) or a soil dwelling earthworm species (*A. caliginosa*, Ac) collected from mesocosms without (−H) and with (+H) herbicide application. (Lt: N = 1–2, Ac: N = 6, mean ± SE). Inset shows a cocoon with a freshly freshly hatched L. terrestris.

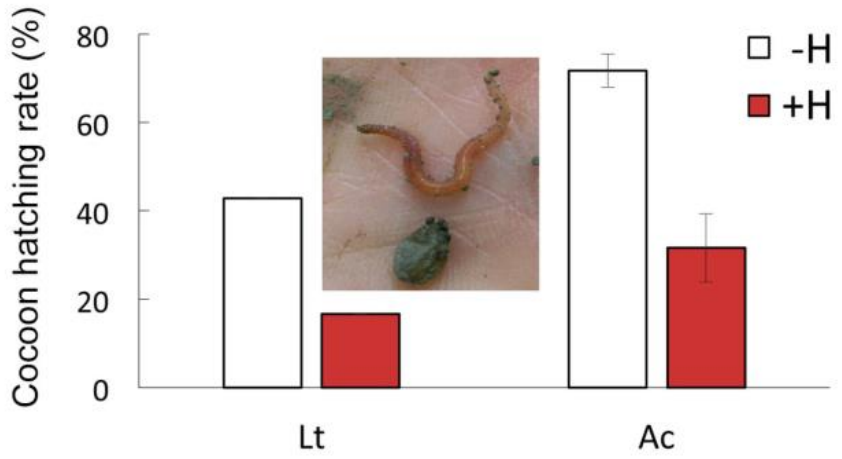
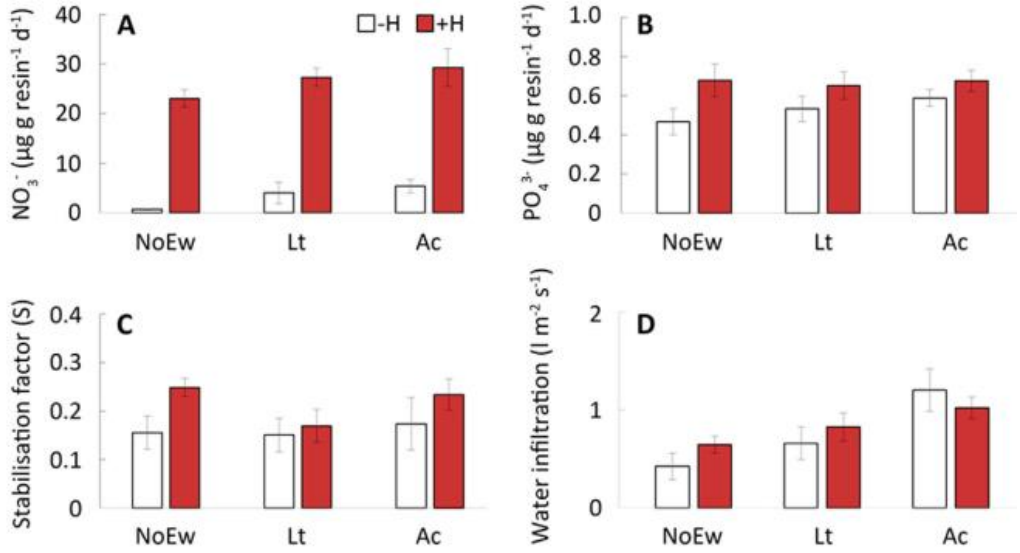


Figure 3. Soil parameters affected by herbicide application (–H, without herbicide; +H, with herbicide application) in response to the presence of different earthworms (NoEw, no earthworms; Lt, *L. terrestris*; Ac, *A. caliginosa*). (**A**) Plant available nitrate (NO^{3-}), (**B**) plant available phosphate (PO_4^{3-}), (**C**) soil stabilisation factor, and (**D**) water infiltration rate. (N = 6, mean ± SE).



Conclusion

Because earthworms play a pivotal role in co-determining how agro- and garden ecosystems function, the observed deleterious side effects of glyphosate-based herbicide application indicate far-reaching consequences of its use in ecosystems. First, the role of earthworms as important ecological engineers in agroecosystems and gardens can be compromised. Reductions in mixing of organic litter within the soil will limit long-term soil microbial activity, effects of earthworms on aboveground herbivores, soil nutrient cycling and availability, all of which may lead to reductions in plant productivity. Second, pulses of nitrate and phosphate availability following herbicide application could increase the risk of leaching or surface runoff of these nutrients into groundwater systems or adjacent aquatic ecosystems, as long as the crop cover is not yet re-established. Although productivity in many agricultural systems depends on the use of pesticides, findings from the study indicate that more serious attention has to be

paid testing pesticides for potential undesirable ecological side effects, especially in light of the projected doubling of global pesticide use by 2050.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: The authors suggest an impact of surface casting production including the number of casts following GBH application in addition to observed increases in soil concentrations of nitrate and phosphate. However, the study provides only supplementary information on the potential outdoor impacts that glyphosate applications could present to surface and burrowing earthworms as it does not appear to be directly relatable to an EU ecotoxicology regulatory risk assessment.
In addition, none of the formulations tested is the representative formulation for the glyphosate EU renewal.

Further points of clarification:
The representative formulation for the glyphosate EU renewal is MON 52276, which has a nominal glyphosate acid equivalent (a.e.) content of 360 g/L, formulated using glyphosate isopropyl-ammonium salt and a quaternary-ammonium based surfactant.

Assessment and conclusion by RMS:

In a greenhouse experiment, the impact of Roundup (different formulations of Roundup applied within a week) on two earthworm species with different feeding strategies.
Weed communities (grass, leguminous herb and non-leguminous herb species) were established in pots.
Vertically burrowing or horizontally burrowing earthworm species were added.
Mesocosms were treated with a "lower-than-recommended" dose of glyphosate-based herbicide.
3 treatments (no earthworms, Lt, Ac) and two herbicide treatments (− H, + H)

The main objective was to investigate the surface casting activity of the worms.

Results:
Vertically burrowing earthworms (Lumbricus terrestris) almost ceased activity three weeks after herbicide application, while the activity of soil dwelling earthworms (Aporrectodea caliginosa) was not affected.
RMS notes that the reduced surface casting activity after herbicide treatment might be that L. terrestris avoided plant residues contaminated with glyphosate on the surface. As a consequence these earthworms might have lived in deeper soil horizons and avoided surface foraging and casting (as hypothetized by the authors). So the relevance of this parameter for the risk assessment is questionable. No quantitative link can be made with the protection goals. RMS further notes that at the end of the experiment, 93.3 ± 6.6% and 86.7 ± 9.9% of introduced numbers of L. terrestris and 100.0 ± 0.1% and 100.0 ± 2.6% of introduced numbers of A. caliginosa in –H and + H treatments were retrieved, respectively. So no significant impact on survival was noted.

Reproduction of the soil dwellers was reduced by 56% within three months after herbicide application. According to the authors, reproduction success of both earthworm species substantially decreased after herbicide application.
25 cocoons from L. terrestris (18 cocoons in two –H, 7 cocoons in one + H mesocosm) and 292 cocoons from A. caliginosa (193 cocoons in six –H, 99 cocoons in six + H mesocosms).
RMS notes that 12 replicates were conducted for each treatment, so it appears that a number of replicates did not provide cocoons even in the non-treated pots. RMS doubts the reliability of the reproduction outputs.

Hatching rate, i.e., percentage of cocoons from which earthworms hatched, decreased from 43% to 17% for L. terrestris (no statistical test was performed because of two few replications among treatments) and from 71% to 32% for A. caliginosa ($P < 0.001$) when cocoons were collected in mesocosms without herbicide or with herbicide treatment, respectively. This seems to indicate an effect on hatching, significant for A. caliginosa.

Herbicide application led to increased soil concentrations of nitrate by 1592% and phosphate by 127%, pointing to potential risks for nutrient leaching into streams, lakes, or groundwater aquifers.

No biological data was presented. Besides RMS notes that the actual exposure cannot be quantified, no concentration measurements available. The application rate is not clear and involves several products applied successively. Weeds were well developed at the time of spraying, so interception occurred but it is not known to what extent.

These uncertainties make this study not reliable.

The study is considered less relevant but supplementary (uncertainty regrading formulation) and not reliable.

Data point:	CA 8.2.4
Report author	Alhewairini S. S.
Report year	2017
Report title	Toxicity of the herbicide glyphosate to non-target species *Caenorhabditis elegans*.
Document Source	Journal of Food, Agriculture & Environment (2017), Vol. 15, No. 2, pp. 97
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

Full summary of the study according to OECD format

In this study, the effects of glyphosate on *Caenorhabditis elegans* were investigated by looking at feeding inhibition and the mortality of *C. elegans*. Feeding of worms was almost completely inhibited by glyphosate at 12,000 ppm, although glyphosate was clearly becoming toxic, even at 120 ppm. *C. elegan*s mortality was very high (68, 82 and 100%) after exposure to glyphosate at 120, 1200 and 12,000 ppm, respectively. Not only high concentrations (12,000 ppm) but also low concentrations (1.2 ppm) of glyphosate had significant effects in the mortality of *C. elegans* compared with control.

Materials and Methods

C. elegans culture and maintenance
Culture and maintenance of the wild type (N2) *C. elegans* was according to standard protocols. Nematode growth media (NGM) was used to grow worms. The plates were seeded with *Escherichia coli* (*E. coli*) strain *OP50* and incubated at 37 ºC for 24 hours. Both single worms were transferred to new plates by using a sterile platinum pick or chunking of crowded plates by cutting and transferring a small cube of agar from an old NGM plate of *C. elegans* to a new plate using a heat flamed stainless steel scalpel blade. The plates were incubated at 15ºC.

Isolation of eggs
In order to isolate a large number of worms, adult worms were grown on new plates as described above. Plates were washed off by using K-medium into the 50 ml tubes. Tubes were left on ice for 10-15 min to allow the worms to settle to the bottom of the tube. Supernatant was removed from the tube without disturbing the worms and replaced with the egg isolation bleach solution. Tubes were shaken for 7 min to disrupt the worms and trigger release of their eggs, and centrifuged for 30 minutes at 3000 rpm. The supernatant was replaced with fresh K-medium and the process was repeated 3 times to remove any traces of the bleach solution. Tubes were then shaken on a rotary shaker overnight to allow L1 larvae to hatch. The contents of the tubes were allowed to settle and the supernatant was removed leaving 2 ml in the 50 ml tube. The remaining 2 ml was transferred to several NGM agar plates seeded with *E. coli* and incubated at 15 ºC.

Solutions and exposure protocols
After carrying out an egg isolation to synchronize, the cultures of worms were washed off plates using K-medium. The supernatant was transferred to a 50 ml tube using Pasteur pipette. The tube was left on ice for 10-15 min to allow the worm to settle in the bottom of the tube. The supernatant was removed from the tube (without disturbing the sedimented worms) using a Pasteur pipette. Bacterial solution was added to the tube and 750 µl of worm suspension was transferred to a labelled 6-well plate. A dilution series of glyphosate in bacterial solution was prepared by diluting the (100,000 ppm stock solution of glyphosate in water) to give a range from 2.4 to 24,000 ppm. Of bacterial solution 750 µl containing toxicant and bacteria + 750 µl worms and bacterial solution were added to each well to give final concentration of the toxicants from 1.2 to 12,000 ppm. Control wells containing either no worms with the highest concentration of toxicant or no toxicant were included. Each treatment was replicated four times. The 6-well plates were incubated for 1 hour and the content of each well was transferred into a labelled tube using a Pasteur pipette. The tubes were left on ice for 10-15 min for the worms to settle to the bottom of the tube. The supernatant was carefully removed to avoid disturbance of the worms and transferred to a plastic 10 mm cuvette. The OD of the supernatant was measured at 550 nm in a spectrophotometer to measure the concentration of bacteria remaining in suspension. Mortality of worms at different concentrations of toxicant was recorded by direct microscopic observation of samples of the worm suspension

Statistical analysis
The feeding inhibition statistical analysis was done using Microsoft Excel by Dunnett's multiple comparisons tests. The results were expressed as mean OD ± SEM for each treatment. Dilution-response curves for the mortality assay were plotted using Graph pad Prism version 7. Data points were the mean ± SEM of each glyphosate dilution and the graphs were fitted using a nonlinear regression with a four parameter logistic equation where the upper plateau was set to 100% and the lower plateau was set to 0.

Results

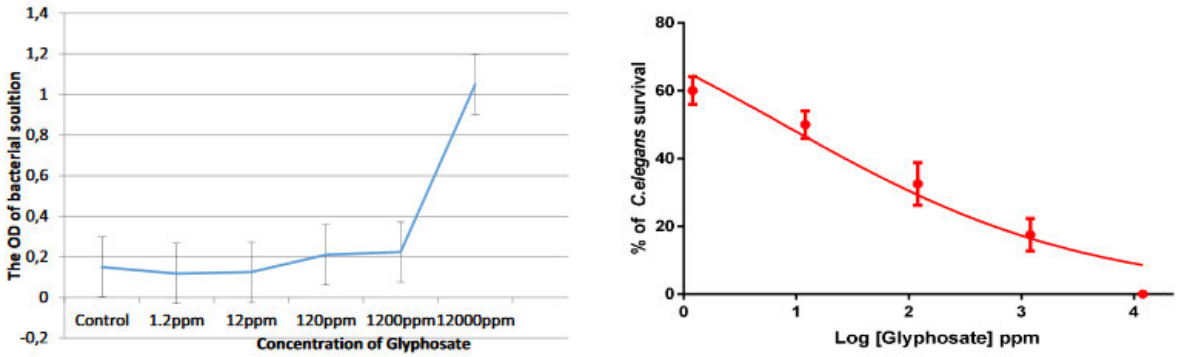
There was a rise in the OD that is associated with an increase in the different concentrations of glyphosate. The results showed that glyphosate becomes toxic either at 1200 or at 12,000 ppm (as shown in Table 1). The increase in the OD was because of the ability of glyphosate to inhibit the feeding of the worms (more bacteria is present leading to an increase in the OD). Table 1 shows the OD (5 replicates) of each sample concentration glyphosate given in ppm. In all cases, the OD of the suspension declines over the period of the experiment (Fig. 1). There was no significant difference between control and any concentration of glyphosate up to 12 ppm after incubating for 30 min (One-way ANOVA $p>0.05$). Dunnett's test indicates that concentrations of 120 ppm and above are significantly different from the control (One-way ANOVA $p<0.05$). Control treatments containing no worms were included in this study to show if glyphosate can produce the change in OD over time in the absence of feeding. Furthermore, the results have shown that no change in the OD was recorded in the control, containing glyphosate with no worms. This step was done to ensure that glyphosate does not produce change in the OD. Under the microscope the number of dead worms was recorded (mortality), however, some of the worms were still alive (completely paralyzed) at lower concentrations whereas at the highest concentration 12,000 ppm no worms were alive (100% mortality) (Table 1, Fig. 1). All glyphosate concentrations 1.2, 12, 120, 1200, and 12,000 ppm tested in this study had significant effect on the mortality of *C. elegans* ($P<0.05$, F test graph pad Prism 7) (Table 1, Fig. 1). Furthermore, no extra assessments were recorded because [1] in most cases, worms feeding were completely inhibited by glyphosate and [2] *C. elegans* mortality was very high at most used concentrations.

Table 1. Dunnet's post hoc test shows statistical differences between control and glyphosate-treated samples of 5 replicates. Note that a non-worm control is not included in the Dunnett's test as it does not confirm any inhibition of feeding after 30 min. & Mortality percentage of *C. elegans* after incubation for 30 min with 1.2, 12, 120, 1200 and 12,000 ppm of glyphosate compared with control (0% mortality).

Dose of glyphosate, ppm	OD ± SEM	Percentage of mortality (%)
0 (control)	0.115 ± 0.008	0
1.2	0.118 ± 0.006 N.S	40
12	0.123 ± 0.009 N.S	50
120	0.210 ± 0.007***	68
1200	0.224 ± 0.033***	82
12,000	1.05 ± 0.008***	100

*** = $p<0.001$ and N.S = no significant difference $p > 0.05$

Figure 1. Left side (blue line): The effect of various concentration of glyphosate on feeding of *C. elegans* (N2) after incubation for 30 min at 20 °C. The columns show the mean OD ± the SEM for 5 replicates. & Right side (red line): Comparison of the effects of five different concentrations of glyphosate after 30 min incubation on the survival of N2 expressed as % of the control survival in bacterial solution. Each point is the mean ± SEM of 5 replicates. The lines were fitted using a non-linear regression in Graph Pad Prism 7 with the maximum plateau as 80% and the minimum as 0%.



Conclusion

The main objective of this study was to see if feeding is affected when exposed to glyphosate by looking at feeding behavior in worms that are consuming bacteria (*E. coli*) as well as check *C. elegans* mortality. Results showed that there is a rise in the OD, which correlates with an increase in the concentration of glyphosate. The increase in the OD indicates that there is decline in the consumption of food because of the effects of glyphosate on the worms (more bacteria are present). This provides evidence for feeding inhibition of these worms, especially at a high concentration of glyphosate. Clearly, the highest mean difference from the control group was attained with 12,000 ppm glyphosate. Dunnett's test indicates that this difference is statistically significant. Moreover, the 120 ppm and 12,000 ppm glyphosate samples were also found to be significantly different from the control group $p<0.05$. It can be considered that glyphosate is becoming toxic at 120 ppm but there is a sharp increase in the OD value at 12,000 ppm. That means extreme feeding inhibition occurred at these concentrations but was strongest with 12,000 ppm. Results showed that 1.2 ppm of glyphosate could produce 40% mortality in *C. elegans* after short time of exposure (30 min). Toxicity of glyphosate can increase after 24 hours of exposure and some toxicity symptoms can appear. The results in this study were taken after 30 min of exposure to glyphosate and no results were taken beyond this time as glyphosate at high concentration was able to completely inhibit feeding and the mortality of *C. elegans* was very high.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: The study has not been conducted according to a recognised test guideline and there are no validity criteria presented. The generated endpoints are not based on direct effects on the nematode, but instead, are based on the optical density related to the density of bacteria present in the NGM ajar test cultures. It is unclear if the presented mortality data were due to direct effects of glyphosate in the cultures, or due to indirect effects associated with reduced feeding activity. There was no test substance information presented and glyphosate concentrations were not measured / confirmed during the study. Finally, there were no quantifiable endpoints presented in the paper, that would be considered applicable to an ecotoxicological regulatory risk assessment for the glyphosate EU renewal.

Assessment and conclusion by RMS:

Caenorhabditis elegans is a 1mm nematode living in soil and feeding on bacteria. From RMS point of view this species is a model organism representing both nematodes and bacteria. Concentrations used are within the environmentally relevant concentrations. On these aspects the study should be further investigated for its appropriatness and relevance for risk assessment purpose.
However there was no test substance information available. Exposure is expressed as glyphosate but the form used was unknown (IPA salt, K salt…) and it cannot be ascertain that commercial formulation (containing surfactants) was not used. Toxicity of glyphosate-based herbicides to non-target organisms vary within a wide range, depending on the surfactant in the product. Therefore RMS considers that this study is less relevant due to uncertainty on the test item.
Besides RMS notes that dose-effect relationship shows an unusual trend. High mortality even at low concentrations were observed and a slow mortality increase between 1.2 ppm (40% mortality) and 1200 ppm (82% mortality) i.e. on a very large exposure range. No reliable endpoint can be derived.
Assuming this species representative of a soil microorganism group, RMS notes that in other studies transitional effects of glyphosate were observed on microbial activity with recovery thereafter. This study does not inform on recovery rate.
Concentrations at which feeding inhibition was affected (120 ppm and above) were above those expected for the representative uses intended. So no critical endpoint would be derived from this parameter.
The study is considered not reliable for regulatory risk assessment purpose.

RMS considered this study as : Less relevant but supplementary (due to uncertainty on the test item) and not reliable.

11. Soil Organisms other than earthworms

Data point:	CA 8.4.2
Report author	Druart C. *et al*.
Report year	2010
Report title	Towards the development of an embryotoxicity bioassay with terrestrial snails: screening approach for cadmium and pesticides.
Document Source	Journal of hazardous materials (2010), Vol. 184, No. 1-3, pp. 26-33

Guidelines followed in study	None
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

Currently no bioassays are available to assess the embryotoxicity of chemicals with terrestrial soil invertebrates. The authors therefore presented a new method for embryotoxicity testing with snail eggs: a relevant biological material that incubates in soil and that can be exposed to contaminants from leachates and soil solution. The effects of aqueous solutions of the herbicide formulation Roundup® and its active substance glyphosate were studied. Endpoints were the hatching success and observations of embryo abnormalities after exposure. Roundup® was found to be more toxic than glyphosate alone ($EC50_{ai}$ = 18 mg/l and about 1300 mg/l, respectively). Abnormalities of embryogenesis in non-hatched embryos depended on the concentration considered.

Materials and methods

Chemicals
Roundup® Biovert 360 (360 g/L glyphosate; Monsanto Europe S.A.) was used. Aqueous solution of glyphosate was prepared with solid glyphosate (99%, Sigma-Aldrich Chimie SARL, Lyon, France, CAS No. 1071-83-6). All dilutions were prepared with demineralized water (pH 6.2), which also served as control. Concentrations of final tests are presented in Table 1. For glyphosate, the repeatability (test 2 with the same concentrations) and reproducibility (test 3 with other concentrations) were tested.

Snails and clutches
Adult *Helix aspersa aspersa* Müller (*syn. Cantareus aspersus aspersus* Müller, 1774 or *Cornu aspersum*) snails (aged between four months and one year) came from our standardized laboratory rearing. To isolate the clutches, 125-ml glass pots filled with damp horticultural compost (SEM NF 44-551, with fertiliser; organic matter: 82%, pH: 6.5) were placed in snail cages the evening. The next morning, the glass pots were isolated to identify the laying snail and to be sure that there was only one clutch per glass pot. The duration of egg laying is between 24 and 32 h. After snails have laid their eggs, the clutches can be removed from the compost. In each clutch, eggs were counted (between 70 and 150 per clutch), washed with tap water at 20ºC to remove soil particles and then deposited on damp blotting paper in Petri dishes until the experiments.

Exposure device
Rapidly after egg laying, each clutch was separated into groups of 8-10 eggs, which were placed in Petri dishes (35 mm ×10 mm). Four layers of paper dampened with 0.8 ml of control or contaminated solutions (Table 1) were laid on the bottom of the Petri dishes (this volume is enough to keep with the humidity until hatching). The eggs were incubated in these dishes at 20 ± 2ºC, 18 h light per day and humidity of 80–90% until hatching (about 14 days for controls). For each compound, between 3 and 6

clutches were exposed (Table 1). Twenty days after the beginning of exposure (to be sure not to omit late hatchlings), the mean hatching success for each concentration was calculated. Results were considered valid if the hatching success of controls was higher than or equal to 70% (average value observed in our laboratory rearing for controls).

As the range of pH values for the different solutions was high (Table 1), the sensitivity of egg development to the pH of the medium was assessed using a range of seven pH values (1, 2, 4, 6, 8, 10, and 12) with 6 replicates each, in demineralized water adjusted with HCl or NaOH.

Table 1. Range of nominal concentrations used for each contaminant and corresponding pH.

Solution	Replicate number	Test number	Range of concentrations	pH
Glyphosate (mg/l)	3	Test 1	225–450–900–1800–3600	2.3 < pH < 3.1
	3	Test 2	225–450–900–1800–3600	
	4	Test 3	1000–1400–1960–2740–3840	
Roundup® (mg/l of glyphosate)	5	Test 1	1.8–9–45–225	4.9 < pH < 6.2

Embryo observation

The main steps of embryogenesis were described in unexposed embryos according to Fol's typology. As the eggs are opaque, the eggshell must be removed to see the embryo, which is enclosed in transparent albumen. Embryos were observed with an inverted microscope or a binocular microscope equipped with a camera. After exposure to the chemicals, non-hatched eggs were examined in the same way to determine the stage at which their embryonic development was interrupted.

Statistical analyses

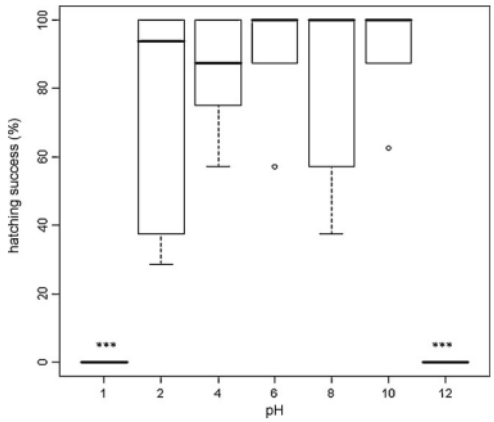
The null hypothesis of independence between exposure and hatching success was tested using the Kruskal-Wallis rank test. When the null hypothesis was rejected, multiple comparisons were performed on ranks using Tukey's Honestly Significant Difference test. All statistics were performed with *R* (2.9.2). The dose-dependent curves and the EC_{50} values were determined with Hill's model using the macro Excel Regtox free version EV6.1.

Results

Hatching success

The influence of pH on hatching success was not significant except for the extreme values, 1 and 12, for which the percentage of hatching was 0% (Fig. 1), with the eggs becoming reddish brown. As pH values of tested solutions were in the range 2.3-6.9 (Table 1), it is likely that the effects observed on eggs are more due to the chemicals than to low pH.

Figure 1. Hatching success of snail eggs exposed to increasing pH. Significant differences ($p < 0.001$) are indicated by ***.

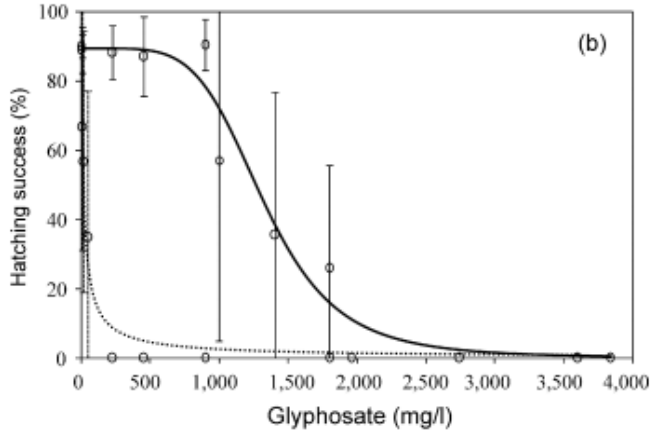


The toxicity of glyphosate was lower than of Roundup® (Table 2 and Fig. 2). Roundup®, at the same concentration as glyphosate, was almost 100-fold more toxic than glyphosate alone (Table 2). Indeed, Fig. 2 shows that, from 225 mg glyphosate/L in Roundup®, hatching success was totally inhibited whereas, for glyphosate alone at the same concentration, hatching success was equivalent to that of the controls.

Table 2. Toxicity (EC_{10} and EC_{50}) of the different contaminants on the hatching success of snail embryos and recommended concentrations for agricultural use. [a] Tests 1 + 2 + 3 were run with all exposure concentrations of each test and all replicates summed.

Contaminant	Test	NOEC	EC10 (CI 95%)	EC50 (CI 95%)	Agricultural recommended concentrations
Glyphosate (mg/l)	Test 1	1800	1262 (830–1822)	1840 (1210–2140)	3600
	Test 2	1400	732 (438–1387)	1190 (910–1530)	
	Test 3	1800	1451 (912–1500)	1580 (1020–1640)	
	Test 1+2+3[a]	1000	854 (685–1348)	1324 (1087–1574)	
Roundup® (mg/l of glyphosate)	Test 1	45	1(0.02–31)	18(2.8–66)	3600

Figure 2. Concentration-response curves obtained by Hill's model after 14 days of exposure of *Helix aspersa* eggs to glyphosate (test 1 + 2 + 3, solid line) and Roundup® (dotted line).



Normal and disrupted embryogenesis

Embryos exposed to glyphosate were blocked late in their development, in a larval stage corresponding to 12 day's development in controls.

Conclusion

Glyphosate and its formulation or its associated adjuvants were toxic to snail embryos at lower concentrations than the recommended application concentration for agriculture.

At equal concentrations of glyphosate, Roundup® was more toxic than glyphosate alone. Two mechanisms could be evoked: a higher toxicity of glyphosate in the Roundup® due to potentialisation of its effects by adjuvants and/or the toxicity of the adjuvants. It appears necessary to assess the risk of the final product (which will be applied to crops) and not only of the active ingredient individually. More data on the ecotoxicity are also needed for adjuvants, for which ecotoxicological information is scarce or absent from databases.

Effects of glyphosate appeared later in the embryonic development just before hatching.

The present results and methodology enlarge the relatively restricted panel of available tests for assessing the embryotoxicity of chemicals towards terrestrial invertebrates.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Glyphosate active substance and glyphosate-based herbicide formulation Roundup® were tested to compare toxicity to land snails. LC_{50} valued were generated, however based on a new methodology (not to any established guideline). The metholodogy and endpoints generated are not relateable to an EU level ecotoxicological regulatory risk assessment for glyphosate EU renewal.

Assessment and conclusion by RMS:

The aim of this study is to assess the embryotoxicity of chemicals with snail eggs (*Helix aspersa aspersa* Müller (syn. *Cantareus aspersus* asperses Müller, 1774 or *Cornu aspersum*).
The effects of Roundup® Biovert 360 (360 g/l glyphosate; Monsanto Europe S.A.) and glyphosate were studied. Endpoints were the hatching success and observations of embryo abnormalities after exposure.
RMS noted that Roundup® Biovert 360 contains 360 g glyphosate / L as MON 52276.
Roundup® Biovert 360 was found to be more toxic than glyphosate alone (EC50a.i. = 18 mg/l and about 1300 mg/l, respectively).
EC10 of glyphosate to *Helix aspersa* was approximately 854 mg/L glyphosate (685–1348).

RMS questions the relevance of the exposure. Four layers of paper (Quantitative filter paper grade 40 ashless, Whatman) dampened with 0.8 ml of control or contaminated solutions were laid on the bottom of the Petri dishes. Eggs were then placed in these Petri dishes until hatching. However snails lay their eggs in the topsoil (2–5cm depth). Thus the study design may influence what will happen in real conditions. It is agreed that they can be exposed to contaminants deposited on the ground and then leached downwards but the relevance of the aqueous solutions used in this study is questionable and not relatable to EU regulatory risk assessment.

This study nevertheless highlights the need to assess the risk of the final product (which will be applied to crops) and not only of the active ingredient individually.
The study seems well conducted (despite the absence of specific guideline). However there are no details of biological observation reported in the paper. Thus, the LC50 calculation cannot be confirmed by RMS and the study design (paper in bottom of petri dishes) is questionable..
This study cannot be taken into account for the assessment of the active substance glyphosate itself.

This study is relevant (glyphosate tested) or less relevant but supplementary (product). But the uncertainty around the protocol and study design and the absence of detailed results makes the study unreliable by RMS.

12. Soil micro-organisms

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13. Non-target terrestrial plants

Data point:	CA 9
Report author	Mondal S. *et al.*
Report year	2017
Report title	Phytotoxicity of glyphosate in the germination of *Pisum sativum* and its effect on germinated seedlings.

Document No	Environmental health and toxicology (2017), Vol. 32, pp. e2017011
Guidelines followed in study	none
Deviations from current test guideline	not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9) as proposed by applicant: See RMS analysis in RMS comment box	Not relevant by full text

The summary as submitted by the applicant is presented here.

Full summary of the study according to OECD format

The present study evaluated the effects of glyphosate on *Pisum sativum* germination as well as its effect on the physiology and biochemistry of germinated seedlings. Different physico-chemical biomarkers, viz., chlorophyll, root and shoot length, total protein and soluble sugar, along with sodium and potassium concentration, were investigated in germinated seedlings at different glyphosate concentrations. This study reports the influence of different concentrations of glyphosate on pea seeds and seedlings. Physicochemical biomarkers were significantly changed by glyphosate exposure after 15 days. The germination of seedlings under control conditions (0 mg/L) was 100% after 3 days of treatment but at 3 and 4 mg/L glyphosate, germination was reduced to 55 and 40%, respectively. Physiological parameters like root and shoot length decreased monotonically with increasing glyphosate concentration, at 14 days of observation. Average root and shoot length (n=30 in three replicates) were reduced to 14.7 and 17.6%, respectively, at 4 mg/L glyphosate. Leaf chlorophyll content also decreased, with a similar trend to root and shoot length, but the protein content initially decreased and then increased with an increase in glyphosate concentration to 3 mg/L. The study suggests that glyphosate reduces the soluble sugar content significantly, by 21.6% (v/v). But internal sodium and potassium tissue concentrations were significantly altered by glyphosate exposure with increasing concentrations of glyphosate. Biochemical and physiological analysis also supports the inhibitory effect of glyphosate on seed germination and biochemical effects on seedlings.

Materials and methods

Healthy uniform pea seeds were chosen as the test plant and were soaked in distilled water (DW) overnight prior to germination. Before germination the seeds were sterilized using 0.1% mercury chloride for 30 seconds, washed in DW several times to remove excess chemical and then dried to eliminate fungal attack. Twenty selected seeds were placed on filter paper inside a sterilized 15 cm Petri plate for seed germination and seedling growth. In each Petri plate 20 mL of glyphosate (Roundup, Marysville, OH, USA) at different concentrations (0.0, 1.0, 2.0, 3.0 and 4.0 mg/ L doses), or DW as the control treatment, was added for 72 hours. Germination and seedling growth were recorded up to 14 days at intervals of 24 hours. The expected environmental concentration 2.6 mg/L, was considered as a realistic value of exposure level, comparable to those mentioned by other authors such as 1.87 mg/L and 3.73 mg/L; higher values have also been estimated (e.g., 10.13 mg/L).

Tissue Sampling

After the experiment (14 days) desirable tissues from glyphosate-treated and control plants were collected. Then tissues were washed in saline solution (0.75%), homogenized in 0.2 M pH 7.4 phosphate buffer (2 mL) and centrifuged (8000 rpm) at 4°C for 25 minutes. The supernatants were kept in Teflon

tubes and stored at -80°C to perform the biochemical analysis of carbohydrate and total protein. Leaves were separated and put in acetone (80%, Sisco Research Laboratories) for the analysis of chlorophyll, i.e., chlorophyll a (Chlo-a), chlorophyll b (Chlo-b) and total chlorophyll (Chlo-t).

Biochemical Analysis

Total Protein
For the estimation of total protein content, 10 mg of plant material was weighed out and crushed first with 10 mL of DW. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 1000 rpm. One milliliter from the supernatant liquid was pipetted out into a test tube, then 5 mL of alkaline copper sulfate reagent was added to each of the test tubes before incubation for 15 minutes at room temperature. Then 0.5 mL of Folin reagent was added and the mixture allowed to stand for 30 minutes. Then the absorbance was measured at 660 nm.

Soluble Sugar
About 1.0 g of plant material was weighed and crushed with a pestle in a mortar with 10 mL of DW. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 10 000 rpm. The supernatant was collected, then 1 mL of this solution was pipette out into a test tube, 4 mL freshly prepared 0.2% anthrone was added to it and the mixture heated in a boiling water bath for 10 minutes at 100°C. This was allowed to cool to room temperature. The absorbance of the developed green to dark green color was measured at 630 nm using UV-vis double beam spectrophotometer (UV-260; Shimadzu, Tokyo, Japan).

Sodium and Potassium
For the estimation of sodium (Na+) and potassium (K+), 0.5 g of dried and ground tissues was digested in Kjeldahl flasks with the addition of an acid mixture of nitric acid (15 mL) and 60% perchloric acid along with sulfuric acid until evaporated to dryness. After that, the residue was cooled at room temperature following dilution with DW (up to 15 mL), filtered with a Whatman No. 42 filter paper and the final volume made up to 50 mL using DW. This process was used for estimation of Na+ and K+ by flame photometer.

Statistical Analysis
Observations were statistically proved using SPSS version 16.0. Probit analysis was performed for percentiles, survival probabilities and cumulative probabilities for the distribution of a stress. Two-way analysis of variance was used to compare mean values of biochemical parameters at different concentrations and differences were considered as statistically significant at p-value <0.05. All biochemical values were represented as mean± standard deviation (SD) (n=10).

Results

Seed germination
Seed germination was negatively correlated with increasing concentration of glyphosate. In this study, 0 and 1 parts per million (ppm) glyphosate concentrations showed the highest percentage of germination, 90 and 75%, respectively, while the germination rate decreased to 55% at 2 and 3 ppm and showed a similar pattern. Treatments at 4 ppm decreased germination to 40%. The results reveal that reticence in pea germination is correlated with the concentration of glyphosate. The entry of water-soluble allelochemicals into the seeds may be the reason for inhibition of germination and growth. On the other hand, the impact of glyphosate on indole-3-acetic acid, the main endogenous auxin in the plant, reduces the rate of germination and growth.
Root Length and Shoot Length
Average root and shoot length showed a regular decrease with increasing glyphosate concentration, reduced to 14.7 and 17.6%, respectively, at 4 ppm. The magnitude of reduction at all concentrations was proportionate to the concentration. The effect of glyphosate is more pronounced in roots and seedlings than matured plants. Glyphosate significantly reduces the activity of acid invertase, hydrolyzing sucrose

to hexose sugars for energy production, which ultimately affects plant growth and maintenance.

Protein and Soluble Sugar

There was a decreasing tendency in total protein content, but it showed a marked (16.05 times) reduction at 4 ppm compared to the control. There was an increase (184.61%) in total protein content at 3 ppm compared to at 2 ppm. Similarly, soluble sugar content also showed a decreasing trend in the control and treatment at 1, 2 and 4 ppm, while the content at 3 ppm (0.44 mg/g) and 2 ppm (0.39 mg/g) was similar. Glyphosate toxicity restrains 5-enolpyruvylshikimic acid- 3-phosphate synthase (EPSP) enzyme activity. EPSP is essential for tyrosine, phenylalanine and tryptophan production, aromatic amino acids associated with protein synthesis and primary and secondary metabolism. Glyphosate and phosphoenolpyruvate (PEP) may perform as competitive inhibitors, which necessitates precursors to amino acid production. Injury to meristematic tissue is attributed to inhibition of branched-chain amino acids in the region. It also affects amino acids from preexisting proteins, which results in a deficiency in the protein store, present in mature tissues. Inhibition of EPSP and PEP activity due to glyphosate toxicity may be responsible for reducing total protein content. The sucrose content is negatively correlated with the activity of soluble acid invertase due to intrusion in photochemical sugar biosynthesis.

Chlorophyll

The chlorophyll content was also reduced significantly ($p<0.05$) at all concentrations compared to the control and showed a regular trend. Both Chlo-a and Chlo-b as well as Chol-t remarkably decreased at 1 ppm, by nearly 90%. This may be due to the breakdown of chlorophyll during stress or inhibition of chlorophyll biosynthesis, a primary symptom of fluoride-induced chlorosis. Consequently, photosynthesis along with the carbon export light period is inhibited by glyphosate although it has no noticeable effect on photosynthesis and the C3 cycle. But glyphosate translocation and inhibition in carbon translocation overlap, validating that glyphosate represses the process of carbon export and eventually disrupts C3 cycle metabolism in bright light, a primary factor in the inhibition of photosynthesis and fast cessation of carbon and glyphosate translocation.

Sodium and Potassium

Glyphosate reduced the Na^+ content and significant ($p<0.05$) differences were detectable with an increase in glyphosate concentration. In contrast, Na^+ content at 2 ppm (3.47 mg/g) and 3 ppm (3.55 mg/g) was approximately the same. Related observations were also noticed for K^+ in treated and control plants. The reduction of K^+ content was significant ($p<0.05$) and less likely to obtain a linear pattern than Na^+.

Conclusion

In conclusion, negative side-effects on plant growth and nutrient status may be caused by glyphosate application at the recommended dosage. The physiological responses showed significantly reduced seed germination, shoot and root length. The chlorophyll content was reduced significantly at all concentrations, which may affect the sugar content. The correlation study revealed that there is a negative effect on Na^+/K^+ content like others. Finally, the maximum inhibitory effect was found in total protein content which may extensively diminish growth and production in addition to the food value of pea seeds.

Assessment and conclusion

Assessment and conclusion by applicant:

Not relevant by full text: Test design of the study is not relevant to EU level ecotoxicological risk assessment for Annex I renewal. In addition, it is not possible to confirm the identity of the test substance used in the study as only 'Roundup, Marysville, OH USA' is mentioned in the methods. Roundup is a brand that contains multiple glyphosate-based herbicides, that contain different co-formulants. Of most importance to the toxicity profile associated with a particular product is whether that product contained polyoxyethyleneamine (POEA) which is not permitted for use in the EU. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal. Moreover, exposure was also not confirmed using chemical analysis.

Assessment and conclusion by RMS:

In this study, 20 seeds were placed on filter paper inside a sterilized 15 cm Petri plate for seed germination and seedling growth. In each Petri plate 20 mL of glyphosate (Roundup, Marysville, OH, USA) at different concentrations (0.0, 1.0, 2.0, 3.0 and 4.0 mg/ L doses. Germination and seedling growth were recorded up to 14 days at intervals of 24 hours.

These concentrations were considered environmentally realistic by the study authors, but this is not explained. RMS is of the opinion that such concentrations are low if it intents to mimic the concentration in the spray. It is not known if this concentration aims to mimic porevater in soil.
Such endpoint cannot be related to the risk assessment for the non-target plants (endpoints being expressed as grammes per ha in the standard EU risk assessment).

Besides, the influence of potential toxic surfactants has not been reported. The formulation is not described and the representativness of the results to assess the toxicity of glyphosate as formulated MON52276 is questionable.

The study is considered not relevant by RMS for risk assessment purpose (due to the uncertainty on the test item and the exposure which is not relatable to the risk assessment).