Key aspects of the information that GRG submitted to address EFSA's request for additional information in the frame of EU glyphosate active ingredient approval renewal, according to Regulation (EC) No 1107/2009

Glyphosate Renewal Group

Regulation (EC) No 1107 /2009, EFSA request for additional information

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Key aspects in the area of toxicology

- **The salivary gland histopathological findings of hypertrophy and increase cytoplasmic basophilia are considered by the applicant as adaptive and non-adverse**
- **A new *in vitro* HPRT according to OECD TG 476 (2016) and *in vitro* micronucleus according to OECD TG 487 (2016) were performed with glyphosate and submitted in the AIR5 dossier. The conclusion is that glyphosate is devoid of genotoxic potential.**
- **Lack of carcinogenic effects attributable to glyphosate**
- **Absence of any effect of glyphosate on sperm morphology**
- **Lack of effect on corpora lutea number support the lack on effect on oocyte development as a whole**
- **No effects on ano-genital distance measured for males and females from the F1-F2 treated animals in comparison to the controls**
- **No endocrine effect of glyphosate**
- **Lack of neurotoxic effects attributable to glyphosate**
- **Genotoxic assays ongoing for N-acetyl glyphosate, N-glyceryl AMPA, and N-malonyl AMPA**

Contents

Summary

The following document summarises points relating to the mammalian toxicology of glyphosate in the environment within the Annex I renewal dossier submitted by the Glyphosate Renewal Group (GRG, applicant). The dossier was evaluated by the Assessment Group for Glyphosate (AGG) acting as RMS consisting of the corresponding competent authorities of France, Hungary, The Netherlands and Sweden.

Following the public commenting period on the draft Renewal Assessment Report (dRAR) from 23rd September to 19th November 2021, an additional information request was received from EFSA on 14th March, 2022 in the context of 'stop-the-clock'. The requests referred to the following areas:

- Position papers to further assess, support and elucidate experimental studies.
- Summary of additional public literature articles.
- Update of study information (e.g. request of historical control data, trend test etc).

The following provides targeted summaries of important topics for the ongoing EU Review process regarding mammalian toxicology.

Salivary Gland Endpoint

Eleven 13-week dietary rat toxicity studies were originally submitted by the applicant and reviewed in the dRAR, and only one study observed salivary gland histopathological findings. The salivary gland histopathological findings of hypertrophy and increase cytoplasmic basophilia are considered by the applicant as adaptive and non-adverse, as summarized below. Therefore, the applicant proposes that a different endpoint, and not the salivary gland, should be selected for the AOEL calculations.

Furthermore, additional data was provided which includes a pathology peer review of the salivary gland microscope slides from the 104-week chronic study in rats and the 13-week study in rats, with direct comparison to the salivary gland findings in the Chan and Mahler (1992) NTP report. The Chan and Mahler study confirms the salivary gland findings in other repeated dose studies in rat or mice are consistent with the findings in other submitted studies. However, these salivary gland effects should be considered non-adverse, and not applicable to human health risk assessment, as summarized below and in the cited documents. Therefore, a different endpoint should be selected for ADI calculations.

Several technical documents were provided to address EFSA request 24 regarding the relevance of salivary gland effects in rodents. A detailed applicant response included a pathology peer review of salivary glands ([redacted], 2022) which directly evaluated the 13- week dietary rat study (CA 5.3.2/001) and 2-year dietary rat study including 1-year interim sacrifice (CA 5.5/007-009), together with a re-reading of the salivary gland slides from a contemporary NTP report (Chan & Mahler, 1992). In addition, an expert white paper was submitted ([redacted] 2022) which addressed salivary gland effects with discussion on considering these effects an adaptive response rather than adverse. A technical symposium addressing salivary gland effects was also included at the virtual EUROTOX 2021 meeting, and a recording together with presentations are publicly available on www.glyphosate.eu. This is consistent with the current International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) for salivary gland toxicological pathology guidance (Nolte *et al.*, 2016).

Genotoxicity

The applicant provided comments on the documents submitted during the public consultation by SumofUS (attachments PCSF-199401) including applicant's comments on the adequateness of the submitted glyphosate database and on the HEAL document PCSF-203400 and applicant's explanations on the reason why no further *in vivo* studies investigating effects of glyphosate on DNA have been conducted.

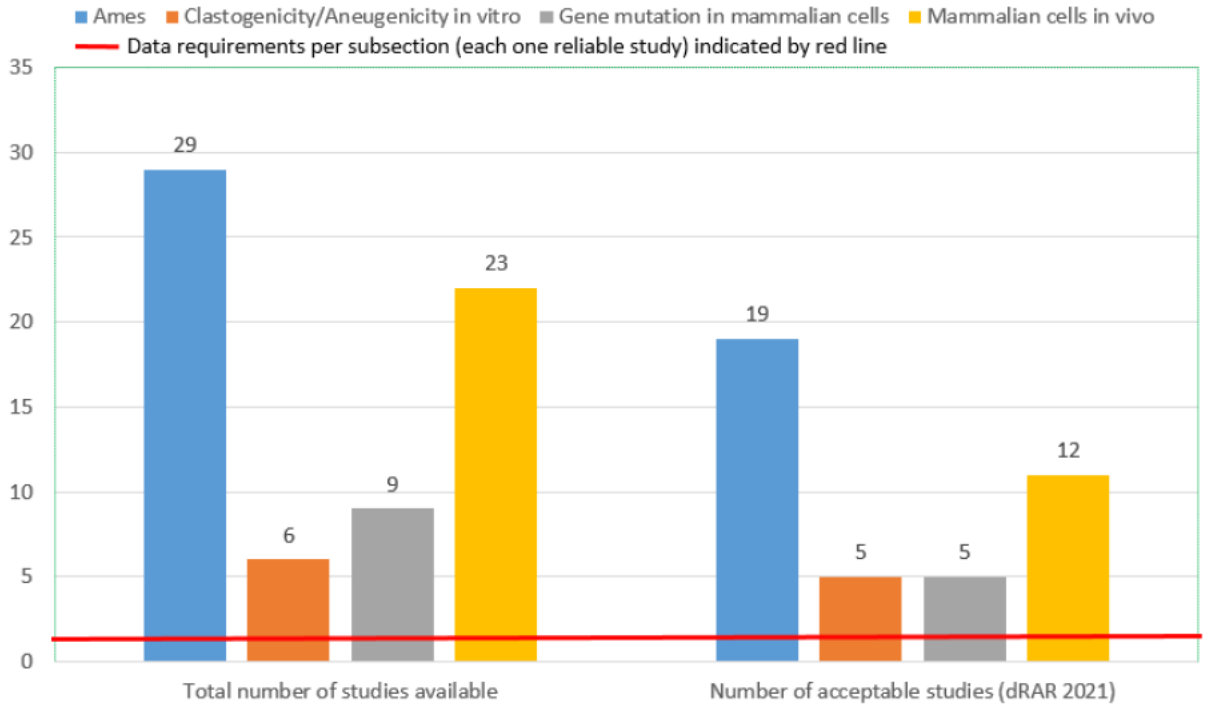
Many of the OECD testing guidelines for genotoxicity were updated and new requirements adopted in 2016. Therefore, the applicant has carried out new studies to fill the potential data gap for gene mutation in mammalian cells and aneugenicity. **A new *in vitro* gene mutation in mammalian cell (HPRT) according to OECD TG 476 (2016) and *in vitro* micronucleus according to OECD TG 487 (2016) were performed with glyphosate and submitted in the AIR5 dossier. These studies have been assessed by the Assessment Group on Glyphosate (AGG) and the evaluation of these good quality studies is available in the dRAR under points B.6.4.1.40 and B.6.4.1.41.**

These new studies (fully reliable) complete the requirement for genotoxicity evaluation under EC 1107/2009:

- Bacterial assay for gene mutation (Ames test)
- One test on clastogenicity/aneugenicity in mammalian cells (e.g. Micronucleus test *in vitro*)
- One mutagenicity test in mammalian cells (HPRT or MLA)
- One *in vivo* test (e.g., Micronucleus test *in vivo)*

Overall, more than one acceptable and supportive study is available for each of the different endpoints for genotoxicity assessment, as indicated in the Figure below.

As no indication of mutagenic properties was observed in any of the *in vitro* studies nor in the *in vivo* micronucleus test, no further *in vivo* testing is triggered.



*Overview on the total number of available genotoxicity studies as well as the number of studies assessed as acceptable in the dRAR 2021 by RMS. The minimum number of studies, which is **one per subsection (red line)**, required by Commission Reg. (EU) No 283/2013 is indicated as red line. For all subsections studies are available that comply with the current OECD TG requirements. The numbers above the bars indicate the number of respective studies.*

In the HEAL document PCSF-203400 the authors claimed that the studies submitted by the applicant do not assess fully the genotoxic potential of glyphosate and that in terms of regulatory requirements, two studies are missing, i.e., the transgenic rodent (TGR) somatic and germ cell gene mutation assays, (OECD 488, they report 2013, but there is an update version dated 2020) and a comet assay (OECD TG 489, 2016).

However, in terms of the regulatory requirement and taking into account the EFSA Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment (2011) and clarification of some aspects related to genotoxicity assessment (2017), the TGR or the comet test are follow-up studies to be carried out for substances positive in the *in vitro* tests. As glyphosate *in vitro* reliable genotoxicity studies were clearly negative, further *in vivo* studies are not triggered.

Overall, all the data available on glyphosate genotoxicity, including old and new studies submitted by the applicant and those found in public literature have been considered using a weight of evidence approach to draw the conclusion that glyphosate is devoid of genotoxic potential.

Conclusion on the genotoxic potential of glyphosate

The genotoxic potential of glyphosate has been assessed in the dRAR based on all the available information submitted by the applicant and published in the peer-reviewed scientific literature.

The document and analysis from EFFAT and HEAL criticising the adequateness of the applicant database of glyphosate genotoxicity is not complete as they did not include the current new studies with glyphosate or its metabolite AMPA which were assessed in the dRAR.

In conclusion, none of the studies and/or information cited by HEAL and SumofUS is new or unassessed and all relevant information has been submitted by the applicant.

Long-term toxicity and carcinogenicity

Several follow up requests from the RMS, regarding additional epidemiology and other related scientific literature were addressed by the applicant during stop the clock, including EFSA requests 26, 27, 28, 36, and 47. Summaries of six epidemiology meta-analyses, three epidemiology review papers, one exposure assessment, and one animal paper were prepared. Overall, these papers do not add significant information to the weight of evidence evaluation due to either their low relevance or low to moderate reliability for human health risk assessment. In addition, an independent expert in epidemiology evaluated the group of cancer types known as non-Hodgkin's lymphoma (NHL), ([redacted] 2021). The overall conclusions, below align with the draft Renewal Assessment Report, which together with the May 30, 2022, ECHA CLH conclusion, clearly demonstrates a lack of carcinogenic effects attributable to glyphosate.

From the linked [redacted] 2021 State of the Science review:

Meta-analyses

Meta-analysis involves taking a weighted average of the results of studies on a particular topic. A number of meta-analyses have been published for glyphosate and NHL including Schinasi & Leon (2014), Chang & Delzell (2016), and Zhang *et al*. (2019). These meta-analyses are not primary data.

Rather, they averaged the primary data to increase statistical precision for risk estimates. In a literature mostly populated by low quality case control studies, these meta-analyses are averaging results of questionable validity – reducing random error, while incorporating systematic error in their meta-RR calculations. Accordingly, the meta-RRs and 95% CIs are not interpretable at face value (Greenland 1990) and do not add appreciably to the evaluation of the individual studies regarding glyphosate and NHL.

Meta-analyses can also be out of date as the literature progresses. The update by Andreotti *et al*. (2018) was not included in the meta-analyses by Schinasi & Leon (2014) and Chang & Delzell. In addition, the pooled reanalysis of the North American case-control studies by Pahwa *et al*. (2019) estimated the OR for any use of glyphosate and NHL overall to be 1.1, which indicates that the ORs of 2.1 or 1.9 (second stage model) from DeRoos *et al*. (2003) are likely not valid inputs for calculating a meta-RR as was done in the three available meta-analyses.

Summary & Assessment

With reference to the quality criteria only one epidemiologic study – Andreotti *et al*. (2018) – is judged to be high quality on all counts. NHL cases were histologically confirmed, the prospective design obviated recall bias with respect to self-reported pesticide exposures, lost-to follow-up was minimal obviating concern about selection bias, the numbers of subjects were ample for analyses of NHL by quartiles of exposure, and control for confounding factors was comprehensive. In addition, the frequency of exposure for Agricultural Health Study (AHS) study participants (median 48 lifetime days and a highest exposure quartile of ≥ 109 days) far exceeded that of any of the other studies, most of which defined exposure as 1 day or more in a lifetime. This study is clearly on a much higher quality level than the other studies in the glyphosate NHL literature.

Reproductive toxicity

During the Stop the Clock period the applicant was asked to provide additional information to support the assessment of the potential of glyphosate on reproductive and developmental toxicity, addressing EFSA requests 37-43. Overall, the requests considered 4 main areas:

1. approach followed for the literature review,
2. setting the appropriate NOAELs in the reproductive toxicity studies,
3. comprehensive historical control data to assess the relevance of certain findings in the developmental toxicity studies
4. assessment of the observed incidence of retro-oesophageal right subclavian artery in the rabbit developmental toxicity study in the context of a Non-monotonic dose response NMDR effect.

General considerations on the approach followed for the literature review

The applicant has detailed the approach used for the literature review, in particular with respect to the relevance and reliability criteria applied for reproductive and developmental toxicity endpoints as well as further justification why some studies were not considered.

- For publications related to reproductive and developmental toxicity besides the characterization and description of the test material, assessing the quality of the method was considered critical. In particular when the investigations did not follow an OECD TG guideline. Therefore, the applicant checked whether sufficient detail was provided of the experimental design such as number of animals per dose group, controls, suitability of study duration, housing conditions, the mode of application of test item to animals (stability, vehicle used, route of administration, dosing intervals).
- Concerning the test system, the applicant checked whether an appropriate animal species and strain was selected, taking into account also animal-to-human concordance of developmental and reproductive toxicity and spontaneous incidence of effects in controls.
- The suitability of sampling method, sampling times and procedures for assessing potential reprotoxic effects was assessed. This is particularly relevant when biochemical and functional measurements (like hormone analysis) are reported: validation and suitability of the analytical procedure for hormones/other biomarkers as well as sampling time should be reported.
- The applicant verified whether information on maternal toxicity and/or other maternal clinical effect was adequately reported to understand the appropriateness of the dose levels tested. This because when excessive maternal toxicity occurs, pathways of elimination and metabolism may change dramatically, and the potential observed developmental effects are likely not related to the administered test material but secondary to unspecific unbalanced homeostasis.
- Developmental effects must be well described, and their incidence clearly reported in tabulated forms rather than graphs or plots or even photos which may confound in the assessment of the severity of the effects.
- Finally, accessibility of raw data should be provided, in particular when the investigations were not conducted under Good Laboratory Practice (GLP) conditions.

Unfortunately, almost all of the publications analysed did not fulfill most of the above criteria and for this reason a large number of publications were not suitable for assessing glyphosate effects on the reproductive and developmental toxicity.

Assessment of reproductive toxicity studies to set the appropriate NOAELs

The effects of glyphosate on reproductive toxicity have been investigated in a large number of two-generation studies in the rat which were submitted by the applicant. Six of the submitted studies were considered to be either fully valid or supplementary according to the current OECD criteria for this type of studies. Many publications on glyphosate reproductive and developmental effects retrieved from the literature search have been also considered depending on their relevance and reliability.

During the stop the clock period the applicant addressed additional endpoints included in this and other requests to support setting the overall NOAELs by considering all the reproductive toxicity studies relevant for glyphosate assessment (those concluded to be reliable).The advantage of having multiple studies assessing the same endpoints allows a better understanding of the relevance of the findings based on the consistency of the effects when deriving the overall NOAELs for parental and offspring toxicity as well as for reproductive toxicity.

An example of this would be the lower number of homogenization resistant spermatid present in the cauda epididymis observed in F0 generation males at 15000 ppm (309 million/gram compared to 400 million/gram in control) of study B.6.6.1/01-3 should be considered to be at most, an equivocal effect given the lack of effect on any other sperm endpoint and in the absence of any histopathological change in the male reproductive organs. Furthermore, sperm from male rats given 30000 ppm were evaluated in another study (B.6.6.1/05). No adverse effects on sperm were reported for males receiving this higher dose level which also included the enumeration of homogenization-resistant spermatids from the cauda epididymis. This was further confirmed following re-assessment of the sperm parameters in study B.6.6.1/05. The laboratory experts retrieved the archived raw data from this study, checked the data and compiled an updated table with the incidences of sperm morphology observations and confirmed the absence of any effect of glyphosate on sperm morphology.

The applicant considers that the lack of effect on fertility in the F0 generation of study B.6.6.1/05 is also relevant to the decision on potential effects on reproductive toxicity as there were no treatment-related effects on sperm parameters or on microscopic examination of the reproductive organs. The slightly reduced number of F1 fertile pairings likely occurred by chance taking into account the lack of effect on fertility or litter size at 30000 ppm in another study (B.6.6.1/10).

Further clarification regarding potential effects of glyphosate on reproductive parameters in the females was included in the request of historical control data on the number of large follicles observed in the ovaries at the top doses of F1 females of the reproductive toxicity study B.6.6.1/01. In this study there was a statistically significant higher number of large follicles (38%) for 15000 ppm females ($p<0.01$) when compared to control. The applicant could contact the testing facility and ask to provide the appropriate HCD to support the data of this study carried out in 2007. However, there were limited historical control data available due to the change to a different animal strain subsequent to 2007. The number of oocyte count in the animals treated with glyphosate seem higher than the normal background. Overall, the elevation in large oocyte counts for high dose animals is not considered to be a treatment-related but a chance result, because there was no indication of an effect on oocyte maturation as all cell types were represented in the slides. Large follicles do become the corpora lutea and there were no effects on corpora lutea number. Therefore, the lack of effect on corpora lutea number support the lack on effect on oocyte development as a whole.

In setting the NOAEL for offspring, the delayed sexual maturation (preputial separation) observed in F1 male offspring at 15000 ppm (time at completion: 45.9 day compared to 43.0 day in control) in the most recent study (B.6.6.1/01) should be anyhow considered in conjunction with data from study B.6.6.1/04. Although in study B.6.6.1/01 the top dose was higher, e.g. 15000 compared to that in study B.6.6.1/04, e.g. 10000 ppm, the achieved intake was rather similar, e.g. 1063 mg/kg bw/day in study B.6.6.1/01 *vs.* 985 mg/kg bw/day in study B.6.6.1/04. In both studies the top dose induced effects in body weights more marked in study B.6.6.1/04, but without effects on preputial separations. Taking int account that both *in vitro* and *in vivo* studies investigating the potential androgenic and anti-androgenic effects of glyphosate summarized in appendix E and I, the preputial separation observed at the top dose of study B.6.6.1/01 is very equivocal and unlikely to be related to an endocrine effect of glyphosate.

Further measurements of the ano-genital distance (AGD) values normalized to the cube root of pup weight were carried out to address the request to calculate this parameter more appropriately. The raw data of the study were still available, so they were used to perform a retrospective assessment of anogenital distance of F1-F2 offspring using cube root transformation of pup body weight at Day 1 post-partum (1pp). Results confirm the previous conclusion presented in the study report: there were no effects on ano-genital distance measured for males and females from the F1-F2 treated animals in comparison to the controls.

In conclusion the following are considered to be appropriate value (expressed in mg/kg bw/day) to set the NOAELs for

1. Adult toxicity: 985 mg/kg bw/day (~10000 ppm) based on clinical signs, reduced body weight, increased liver and kidney weight observed in three studies at dose levels equivalent to 1063, 1983 and 2150 mg/kg bw/day. The effect on salivary gland is considered to be adaptive and not adverse.
2. Offspring toxicity: 668 mg/kg bw/day (~10000 ppm) based on reduced body weight from doses equivalent to 985 mg/kg bw/day and the delay on developmental landmark in males at 1063 mg/ kg bw is considered equivocal
3. Reproductive toxicity: 985 mg/kg bw/day (~10000 ppm) based on equivocal effect on sperm parameter at 1063 mg/kg bw/day).

An overview of the key results from the reliable reproductive studies with glyphosate to be used to set the relevant NOAELs is provided in the table overleaf.

Proposed overall NOAELs by considering all the reliable studies. Those conclusions in **bold** from the RMS were re-discussed and challenged by the applicant in the specific requests submitted during stop the clock period. Under column "Overall" the assessment and values proposed by the applicant are presented.

Study	B.6.6.1/01-3	B.6.6.1/04	B.6.6.1/05	B.6.6.1/07-8	B.6.6.1/10	Overall
LOAEL						
parental	15000 ppm (1063 mg/kg bw/day) increased liver & kidney weights		30000 ppm (2150 mg/kg bw/day) clinical signs, reduced body weights, increased liver &kidney weights, reduced prostate weight, distension of caecum	**3000 ppm (197 mg/kg bw/day) changes in salivary glands**	30000 ppm (1983 mg/kg bw/day) clinical signs, reduced body weight	LOEL based on effects on body and organ weights Salivary glands changes were adaptive and not adverse.
offspring	**15000 ppm (1063 mg/kg bw/day) delayed preputial separation**	10000 ppm (985 mg/kg bw/day) reduced pup body weight	30000 ppm (2150 mg/kg bw/day) reduced pup weights, distension of caecum		30000 ppm (1983 mg/kg bw/day) reduced pup weight	LOAEL based on effect on pup weight and caecum distension as delayed preputial separation equivocal and not confirm in other studies
reproductive	**15000 ppm (1063 mg/kg bw/day) reduced number of homogenization resistant spermatid in cauda epididymis**		**30000 ppm (2150 mg/kg bw/day) lower fertility indices**		**30000 ppm (1983 mg/kg bw/day) equivocal reduction in litter size**	LOAEL on reduced number of homogenization resistant spermatid in cauda epididymis is equivocal. Equivocal effects on fertility indices
NOAEL						
parental	5000 ppm (351 mg/kg bw/day)	10000 ppm (985 mg/kg bw/day)	6000 ppm (417mg/kg bw/day)	**1000 ppm (66 mg/kg bw/day)**	10000 ppm (666 mg/kg bw/day)	10000 ppm 985 mg/kg bw/day
offspring	**5000 ppm (351 mg/kg bw/day)**	3000 ppm (293 mg/kg bw/day)	6000 ppm (417mg/kg bw/day)	10000 ppm (668 mg/kg bw/day)	10000 ppm (666 mg/kg bw/day)	10000 ppm 668 mg/kg bw/day
reproductive	**5000 ppm (351 mg/kg bw/day)**	10000 ppm (985 mg/kg bw/day)	**6000 ppm (417mg/kg bw/day)**	10000 ppm (668 mg/kg bw/day)	**10000 ppm (666 mg/kg bw/day)**	10000 ppm 985 mg/kg bw/day

Assessment of developmental toxicity studies

To better understand the relevance of the effects observed in the developmental toxicity studies in the rats and in the rabbit, the applicant was asked to provide and include appropriate Historical control data (HCD) on developmental toxicity parameters, including the incidences of malformations, variations and implantation losses for the developmental toxicity studies as considered necessary based on all comments made on the dRAR.

For the rat, all studies together with the supplementary HCD demonstrate that glyphosate does not induce foetal malformation and the majority of studies demonstrate that foetal development is not impaired at the limit dose of 1000 mg/kg bw/day.

For the rabbit, all studies together with the supplementary HCD demonstrate that glyphosate does not adversely affect foetal viability and neither does it induce foetal malformation. Some minor perturbations of skeletal ossification, considered to be a consequence of the slightly reduced foetal body weight at 300 mg/kg/day were observed but these did not include lumbar ribs.

Non-monotonic dose response (NMDR) of retro-esophageal right subclavian artery in the rabbit developmental toxicity study

The request for the retro-esophageal right subclavian artery to be considered with reference to the EFSA Opinion on the impact of non-monotonic dose-response on EFSAs human health risk assessments (EFSA Journal 2021;19(10):6877) is not understood for the following reasons:

1. The EFSA opinion states that observations of NMDR are particularly relevant for receptor-mediated effects. There is no indication in the publication to suggest that observations of NMDR are relevant to foetal malformations or how it can be determined that these are receptor-mediated effects.
2. If it is considered that observations of NMDR are relevant to foetal malformations, is it the foetal or litter incidence that is relevant? The litter is the statistical unit for evaluation according to the test guideline.
3. The EFSA opinion states that evidence for non-monotonicity of apical effects should be assessed in terms of statistical rigor and biological plausibility. How can this be achieved for low frequency foetal observations and without knowledge of the mechanism of action?
4. Why is the request made for retro-oesophageal right subclavian artery only? This observation refers to a variation in positioning and not to a malformation with an adverse effect on function which may be incompatible with life. Also, the litter incidence is no greater than 1 and cannot therefore be assessed in terms of statistical rigor and biological plausibility as required for the determination of NMDR.
5. In terms of biological plausibility, it would be more appropriate to consider the heart and associated vessels as a potential target organ.

Neurotoxicity

A number of scientific literature papers identified in the public commenting period were reviewed by the applicant. Summaries were prepared and submitted during stop the clock in April 2022 for additional consideration by the RMS and EFSA. The applicant's assessment of these papers concluded that both ECHA and the draft RAR correctly assessed that glyphosate is not neurotoxic.

Two new papers providing a systematic review of public literature on glyphosate and neurotoxicity were published in 2022 and are publicly available. Chang *et al*., 2022, addressing epidemiology studies, and Moser *et al*., 2022, addressing animal literature, were published after stop the clock. Overviews and conclusions of these expert

review papers were presented during a symposium at the recent International Congress of Toxicology on 19 September 2002, in Maastricht, NL. A recording of this symposium is accessible, along with copies of the presented slides, at www.glyphosate.eu

- Chang *et al*.; Systematic literature review of the epidemiology of glyphosate and neurological outcomes. Int Arch Occup Environ Health. 2022 May 23. doi: 10.1007/s00420-022-01878-0.

Abstract

Purpose

Human health risk assessments of glyphosate have focused on animal toxicology data for determining neurotoxic potential. Human epidemiological studies have not yet been systematically reviewed for glyphosate neurotoxicity hazard identification. The objective of this systematic literature review was to summarize the available epidemiology of glyphosate exposure and neurological outcomes in humans.

Methods

As of December 2021, 25 eligible epidemiological studies of glyphosate exposure and neurological endpoints were identified and assessed for five quality dimensions using guidance from the U.S. Environmental Protection Agency. Studies that assessed personal use of glyphosate were prioritized, whereas those assessing indirect exposure (other than personal use) were rated as low quality, since biomonitoring data indicate that indirect metrics of glyphosate exposure almost always equate to non-detectable glyphosate doses.

Results

Overall, the scientific evidence on glyphosate and neurotoxicity in humans is sparse and methodologically limited, based on nine included epidemiological studies of neurodegenerative outcomes (two high quality), five studies of neurobehavioral outcomes (two high quality), six studies of neurodevelopmental outcomes (none high quality), and five studies of other and mixed neurological outcomes (one high quality). The five high-quality studies showed no association between glyphosate use and risk of depression, Parkinson disease, or peripheral nerve conduction velocity. Results were mixed among the eight moderate-quality studies, which did not demonstrate consistent associations with any neurological endpoints or categories. Low-quality studies were considered uninformative about possible neurotoxic effects due primarily to questionable assessments of indirect exposure.

Conclusions

No association has been demonstrated between glyphosate and any neurological outcomes in humans. To move the state of science forward, epidemiological studies should focus on scenarios involving direct and frequent use of glyphosate while collecting information on validated health outcomes, concomitant agricultural exposures, and relevant personal characteristics.

- Virginia C Moser, Keith Morris-Schaffer, Jason R Richardson & Abby A Li (2022) Glyphosate and neurological outcomes: A systematic literature review of animal studies, Journal of Toxicology and Environmental Health, Part B, 25:4, 162-209, DOI: 10.1080/10937404.2022.2083739.

Abstract

Studies of nervous system effects of glyphosate, a widely used herbicide, have not been critically examined. The aim of this paper was to systematically review glyphosate-induced neurotoxicity literature to determine its usefulness in regulatory decision-making. The review was restricted to mammalian studies of behavior, neuropathology, and neuropharmacology; in vitro and other biochemical studies were considered supplementary

information. Glyphosate formulation studies were also considered, despite uncertainties regarding toxicities of the formulated products; no studies used a formulation vehicle as the control. Inclusion criteria were developed a priori to ensure consistent evaluation of studies, and in vivo investigations were also ranked using ToxRTool software to determine reliability. There were 27 in vivo studies (open literature and available regulatory reports), but 11 studies were considered unreliable (mostly due to critical methodological deficiencies). There were only seven acceptable investigations on glyphosate alone. Studies differed in terms of dosing scenarios, experimental designs, test species, and commercial product. Limitations included using only one dose and/or one test time, small sample sizes, limited data presentation, and/or overtly toxic doses. While motor activity was the most consistently affected endpoint (10 of 12 studies), there were considerable differences in outcomes. In six investigations, there were no marked neuropathological changes in the central or peripheral nervous system. Other neurological effects were less consistent, and some outcomes were less convincing due to influences including high variability and small effect sizes. Taken together, these studies do not demonstrate a consistent impact of glyphosate on the structure or function of the mammalian nervous system.

Metabolite data

Applicant responses to EFSA requests 50 and 52 are summarized below.

Literature review on metabolites

The literature reviews submitted (see literature review reports KCA 9/001 and 9/002; one for the search period January 2010 – December 2019 and one for the search period January 2020 – June 2020), covers glyphosate and likewise its metabolites. Articles retrieved on metabolites, were assessed for relevance/reliability and summaries were submitted as appropriate.

Assessment of genotoxicity of metabolites

The QSAR report was updated with the corresponding new experimental data on metabolites (please see below table). Based on QSAR and/or subsequent read-across analysis on genotoxicity endpoints on mutagenicity, clastogenicity and aneugenicity were concluded negative for all metabolites of glyphosate where no (or incomplete) experimental data was available (N-acetyl-glyphosate, N-glyceryl-AMPA, N-malonyl AMPA, Methyl phosphonic acid).

Apart from already submitted genotoxicity studies presented in RAR Volume 3 B6.8.1, the following studies were recently conducted or are planned (see overview Table 1).

Table 1: Overview on additional genotoxicity studies planned/submitted for metabolites

Metabolite	Study type	Status	Result
N-methyl glyphosate	Ames	Final; summary and report submitted herewith	Negative
	In vitro MNT	Final; summary and report submitted herewith	Negative
N-methyl AMPA	*In vitro* MNT	Final; summary and report submitted herewith	Negative
N-acetyl glyphosate	*In vitro* MNT	Planned (start 1Q 2023)	N/A
N-acetyl AMPA	*In vitro* MNT	Final; summary and report available. Results: non-genotoxic. Not available by stop the clock deadline. Available on www.glyphosate.eu	Negative
N-malonyl AMPA	Ames	Planned, but delayed due to test material synthesis issues	N/A
	In vitro MNT		
N-glyceryl AMPA	Ames	Study start 1Q 2023	
	In vitro MNT		

Considering the newly provided experimental data, a set of guideline compliant *in vitro* genotoxicity studies assessing potential mutagenicity (Ames), clastogenicity and aneugenicity (*in vitro* MNT) is now available for metabolites AMPA, N-acetyl AMPA, N-methyl AMPA, and N-methyl glyphosate, from which all metabolites are concluded to be non-genotoxic.

Further, for N-acetyl glyphosate, N-glyceryl AMPA, and N-malonyl AMPA studies are planned; bacterial mutagenicity (Ames) assay with the last two and clastogenicity/aneugenicity (*in vitro* MNT) on all three are planned for 1Q 2023, except for N-malonyl AMPA studies which are delayed due to issues in test substance synthesis. Nevertheless, the read-across analysis indicates that all three metabolites are non-genotoxic.

General toxicity

For metabolites AMPA, N-acetyl glyphosate and N-acetyl AMPA acute and subacute toxicity data are available in rats. Moreover, AMPA was extensively investigated for metabolism/excretion, subacute toxicity, for skin sensitisation and developmental toxicity. Data were already presented within CA 5.8.1 (RAR Volume 3 CA B 6.8.1). All metabolites were found to be of low acute oral toxicity (LD_{50} > 5000 mg/kg bw). Further, based on the 90-day study in rats all three metabolites are of similar toxicity than glyphosate.
Overall, it can be concluded that AMPA, N-acetyl glyphosate and N-acetyl AMPA are of similar toxicity as glyphosate and the same reference values can be applied.

Nevertheless, it should be noted that metabolites N-acetyl glyphosate, N-acetyl AMPA, N-glyceryl AMPA and N-malonyl AMPA only formed as plant metabolites in uses on specific genetically modified / tolerant crops which are no longer on the global market, and therefore those metabolites are not relevant for any world region, including the EU.

Other studies

Specifically, on glyphosate and its alleged microbial impacts, including on the gut microbiota, there are several important considerations, addressed by the applicant in responses to EFSA request 48:

- Gastrointestinal endpoints are already considered to be the most sensitive to acute, intermediate, and chronic duration oral exposure of laboratory animals to glyphosate, with the main effects of soft stool or diarrhea. Given that stool consistency is known to be one of the most significant co-variates of fecal microbiome composition (Falony *et al*., 2016 and numerous other publications) it thus not surprising that studies that exceed the NOAELs are likely to result in altered stool consistency, and may thereby also demonstrate altered gut microbiota composition from controls. While some gut microbiota perturbation is theoretically possible due to the direct inhibition of EPSPS at high glyphosate doses, there are numerous other mechanisms, unrelated to the enzymatic target of a substance, by which a relatively poorly absorbed substance such as glyphosate could cause stool softening and diarrhea at high doses that exceed current NOAELs, such as an osmotic or pH effect, and resultant changes in microbiota from the unexposed controls. The pH of glyphosate test solutions have been demonstrated to be a mechanism of toxicity, thus a daily gavage dose, effectively a bolus dose of an acidic substance alone may impact gut microbes ([redacted] 2003) rather than by inhibition of the target enzyme of glyphosate.
    - References:
    - Falony *et al*. 2016
    - Reliability and relevance of cited literature: The previously described limitations in the microbiota field notwithstanding, these are reliable studies in that methods are sufficiently reported and within typical practices for the current state of the field. The relevance of these studies is very significant. Falony *et al*. 2016 provides critical context on the complexity of the human gut microbiota, including the many covariates of this system, as well as that, even after accounting for such a large sample size (from nearly 4000 people), the diversity of the microbiota is still not accounted for.

- There is published evidence that the shikimate pathway, the target pathway of glyphosate, is no longer functionally intact in many host-associated microbes, including members of the gut microbiota. (Zucko *et al*., 2010; Mesnage and Antoniou, 2020). Gut microbes can obtain aromatic amino acids from the intestinal milieu, thus alleviating the need to synthesize them *de novo*, and the selection pressure to maintain this biosynthetic pathway is thereby diminished. Thus, the shikimate pathway in some gut microbial communities is already functionally inhibited, irrespective of exposure to a substance capable of inhibiting remaining functional enzymes. It was also recently demonstrated that the shikimate pathways of the gut microbiota is largely transcriptionally inactive, reinforcing that this pathway is not as critical for the gut microbial community as is commonly assumed (Mesnage and Antoniou, 2020.)
    - References:
    - Mesnage R. *et al*., 2020. Computational modelling provides insight into the effects of glyphosate on the shikimate pathway in the human gut microbiome. Current Research in Toxicology 1 (2020): 25-33. (in comment 2(345))
    - Reliability and relevance of cited literature: The previously described limitations in the microbiota field notwithstanding, these are reliable studies in that methods are sufficiently reported and within typical practices for the current state of the field. The relevance of these studies is significant. If the target pathway of glyphosate is already non-functional due to loss

of functional genes in the pathway, or if, as indicated by transcriptomic studies, that it is otherwise not commonly used by the gut microbiota due to the availability of aromatic amino acids in the intestinal milieu, then this significantly diminishes the theory of glyphosate inhibition of the EPSPS enzyme of gut microbes as a relevant mechanism of toxicity

- As above, even if gut microbes possess an intact shikimate pathway and a functional EPSPS that could theoretically be inhibited by glyphosate at higher concentrations, any such inhibition is capable of being alleviated by intestinal aromatic amino acids naturally present in the gut (Nielsen *et al*., 2018). An *in vivo* study of rats demonstrates minimal changes to the gut microbial community composition even at doses 50 times the EU reference dose (Nielsen *et al*., 2018).
    - References:
    - Nielsen LN *et al*., 2018. Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels. Environ Pollut. 2018 Feb; 233:364-376 (in comments 2(346, 688) and public comment 2(110))
    - Reliability and relevance of cited literature: The previously described limitations in the microbiota field notwithstanding, this is a reliable study in that methods are sufficiently reported and within typical practices for the current state of the field. This is one of the more comprehensive studies on the subject of glyphosate and the gut microbiome of Sprague Dawley rats. The authors report in vitro experiments to determine the sensitivities of different species of bacteria commonly found in the gut microbe samples and demonstrates that aromatic amino acids can alleviate that impact of glyphosate inhibition of EPSPS of an E. coli strain. They also report the results from an in vivo study that exposed groups of rats to 5- or 50-times the EU ADI, with both glyphosate alone or a glyphosate formulation. There were no significant impacts from the treatments at a genus level analysis of gut microbes. They also analysed the aromatic amino acid content in different gut compartments and confirmed their presence, which supports the idea that even if glyphosate inhibits the growth of gut bacteria, there are sufficient amino acids to complement such a deficiency. They did observe significant differences in fecal pH and acetate levels (but not other short chain fatty acids) in the cecum between treatment groups in the 50X ADI test groups and controls. Animals of all test groups have organs that were "physiologically normal." There were 20 animals per test group, housed 2 per cage, with one animal per cage randomly selected for analysis, for a total of 10 animals per test group that were analyzed.

- An example of how the lack of standardized methods to determine environmental compound impacts microbes and microbial communities, including host-associated microbiota, can specifically impact the reliability and relevance of study outcomes. In a recent study on clinically used antimicrobials, authors confirmed that aerobic vs. anaerobic conditions can indeed impact the minimum inhibitory concentrations (MICs) of gut-derived facultative anaerobic bacteria, including that of *Enterococcus faecalis*, where anaerobic conditions led to significantly higher (i.e., less sensitive) MICs (Kovale *et al*., 2021). Similarly, in vitro studies that attempt to find glyphosate MICs of a limited set of pure cultures of gastrointestinal bacteria, vary dramatically depending on the test conditions used. For example, one study reports the MIC *E. faecalis* grown in aerobic conditions as 0.15mg/ml, whereas the MIC for this species was determined by Nielsen *et al*. (2018) to be 40-80 mg/ml under anaerobic conditions that more closely resemble the conditions of the gut. That is, there is up to a 533-fold difference in experimental

MICs for the same species, depending on the experimental conditions tested. As there are no testing standards for determining the MICs of herbicides (and we also note that some of the aforementioned literature on MICs do not do appropriate replication in the experimental determination of MICs), caution should be employed when attempting to interpret the various studies for risk assessment purposes.

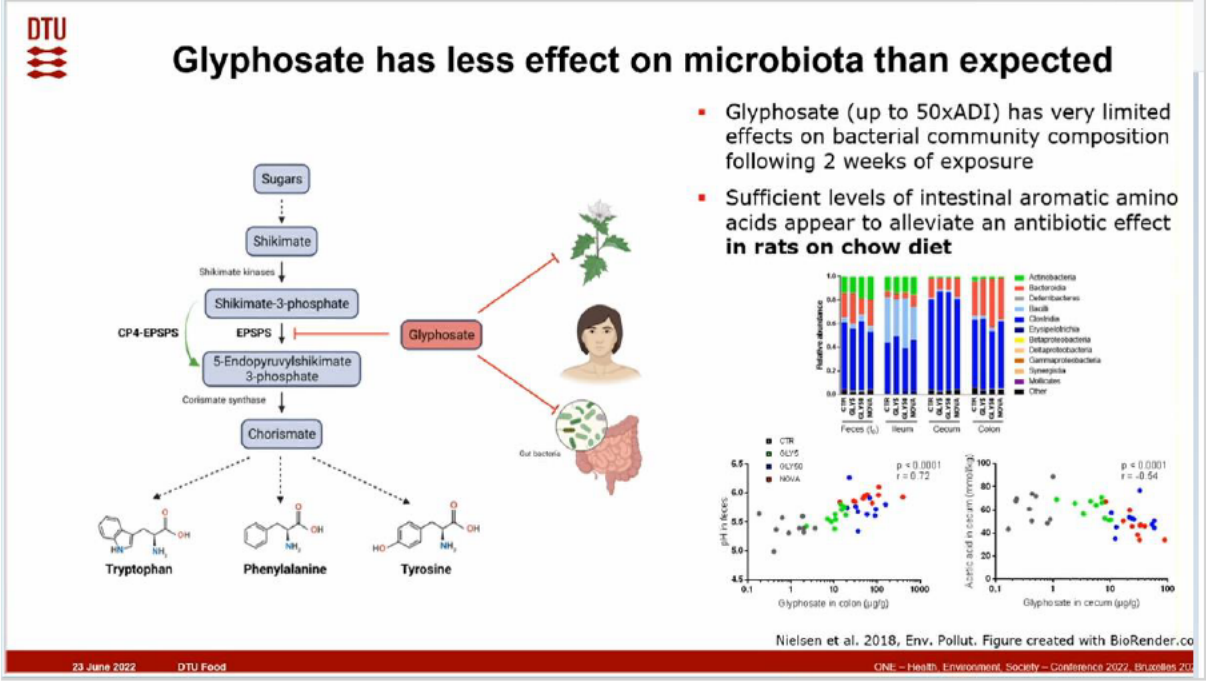
- On the relevance of test doses of microbial, including microbiota, studies with glyphosate. Studies attempting to demonstrate an antimicrobial effect of glyphosate often do not reference the context of their findings in consideration of realistic dietary residue or other exposures. This is a critical consideration as any substance can exert antimicrobial effects at sufficiently high doses. As above, the lowest for MIC reported in the literature is for *E. faecalis* grown in aerobic conditions is 0.15 mg/ml (150 ppm), whereas the MIC for this species was determined by Nielsen *et al*. (2018) to be 40-80 mg/ml (40,000-80,000 ppm) under anaerobic conditions that more closely resemble gut conditions (anaerobic or microaerophilic conditions, with amino acids present). By contrast, the maximum MRL for glyphosate is 500 ppm (or 0.5 mg/ml, the maximal tolerance for animal feed commodities), with actual commonly surveyed residues levels being far lower, e.g., 20 ppm (0.02 mg/ml). Furthermore, tolerances for human food are a couple of orders of magnitude lower than that of feed, with actual residues in food and feed consistently far lower than that which is allowed by MRLs (Vicini *et al*. 2019 and 2021). For example, compiled surveillance and publication data indicates human dietary exposures to glyphosate are maximally 3% of the EU ADI of 0.5 ppm (= 0.5 mg/kg), which equates to 0.015 ppm (or 0.000015 mg/ml) (Vicini *et al*. 2021). Using experimentally derived MICs for *E. faecalis* as an example, the lower (more sensitive) MIC of 0.15 mg/ml (from aerobic conditions that poorly replicate the gut environment) is 10,000 times greater than maximal known human dietary exposures. In the context of *E. faecalis* MICs of 40-80 mg/ml (or 40,000-80,000 ppm) determined from culture conditions that are more representative of the gut, such MICs are $2.67 \times 10^6 – 5.33 \times 10^6$ greater than maximal human dietary exposures. That is, even if these MICs are proven to be valid, there is an accumulating body of evidence that realistic exposures of dietary glyphosate residues for both humans and other animals are several orders of magnitude lower than the proposed MICs of numerous studies (Vinci *et al*., 2021; Vinci *et al*., 2019).
- References:
  - Kovale L. *et al*., 2021. Antibiotic susceptibility of human gut-derived facultative anaerobic bacteria is different under aerobic versus anaerobic test conditions. Microbes and Infection (2021): 104847. (in comment 2(345))
    - Reliability and relevance of this paper: Reliable in both methods used (this is a study using antimicrobials, standards and guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) could be utilized and data reported. Although there are no standards and guidelines for antimicrobial sensitivity testing of environmental chemicals, this study is relevant as it provides critical context and insight as to why there are discrepancies in the literature as to the various outcomes of reported glyphosate minimum inhibitory concentrations (MICs). Studies that use aerobic incubation conditions tend to find lower MICs than those using microaerophilic or anaerobic conditions, which is relevant for gut microbes that exist in microearophilic and anaerobic conditions.
  - Vicini, John L., *et al*. "Residues of glyphosate in food and dietary exposure." *Comprehensive Reviews in Food Science and Food Safety* 20.5 (2021): 5226-5257.

- Reliability and relevance of this paper: This is a comprehensive review that is relevant to addressing glyphosate impacts on the human microbiota in that it compiles published glyphosate residue data, including data from reports on urinary glyphosate residue studies

- Vicini, John L., *et al*. "Glyphosate in livestock: feed residues and animal health." *Journal of animal science* 97.11 (2019): 4509-4518.
  - Reliability and relevance of this paper: This is a review that is relevant to addressing glyphosate impacts on livestock in that it compiles published glyphosate residue data, as well as studies on animal health.

In summary, in the absence of standarized, validated test and analytic methods, and a lack of consensus definitions of healthy microbiome states, consensus toxicity endpoints, or quantifiable endpoints that exhibit dose-dependent properties of adverse health outcomes mediated by the gut microbiota, a typical toxicology reliability and relevance assessment of the literature is challenging and fraught with ambiguities and caveats. It is critical that the focus of safety assessment for a substance remain on quantifiable endpoints that exibit dose-dependent properties adverse health outcomes, which are detected by current animal safety studies.

Shikimate pathway (EFSA microbiota Workshop)



According to Nielsen *et al*. 2018, glyphosate has very limited effects on bacterial community composition following 2 weeks of exposure.

Endocrine Disruptor endpoint

The applicant was requested to review the performed literature search and assess the studies quoted during public commenting. Moreover, a summary of studies considered for ED assessment was to be generated and Appendix E excel file was updated for three publications (as requested under 2(509) of the reporting table referring to RAR, Vol. 1, 3.1.4 List of studies to be generated, still ongoing or available but not peer reviewed, Request 28):

- Ganesan *et al.* , 2020 (KCA 5.6/001, study ID 97)
- Gastiazoro *et al.*, 2020 (KCA 5.8.3/020, study ID 98)
- Xia *et al.*, 2020 (KCA 5.8.3/021, study ID 99)

Following data requirement 2.47, the applicant submitted a summary table on studies taken into account for the WoE assessment on ED and including the published literature cited during the public commenting.

The updated Appendix E Table including Lines of evidence and compiling data on mammalians as well as other non-target organisms, was submitted and addresses EFSA request 47. All changes made by the applicant were highlighted.

Related to this, the applicant also provided a rationale as to why the Transfected Human ERα Transcriptional Activation Assay (KCA 5.8.3/002, study ID 38: OECD TG 455) is considered acceptable, please see below:
17α–methyltestosterone at the highest concentration tested produced a response that was > 40% of the maximally inducing dose of 17β–estradiol in both replicated assays and a more importantly high level of relative transcriptional activity compared to the VC (i.e., 11 and 47-fold induction). These results for 17α–methyltestosterone clearly demonstrate the sensitivity and validity of the ERTA assay conducted for glyphosate to reliably detect a very weak estrogen receptor agonist. In addition, the results from ERTA assay for glyphosate are consistent with the results of estrogen receptor binding assay, uterotrophic assay, female pubertal assay and fish short term reproduction assay and structure activity modeling in the submitted endocrine assessment, which provide multiple lines of evidence that glyphosate is not an estrogen receptor agonist.

Literature data

A position paper describing in more detail a specific part of the approach of the applicant to provide a comprehensive dossier for the renewal evaluation of the active substance glyphosate in the European Union (EU) was submitted. To ensure that all relevant research and scientific information regarding the effects of glyphosate and its metabolites on human health and the environment are included in the dossier, the applicant performed a systematic review of scientific peer-reviewed open literature, following the instructions in the established EFSA guidance. The approach followed is described in detail in this document and additional specific requests received from EFSA and other stakeholders during the public consultation are also addressed.

References

1. Response to Request Public 4
   KCA 5.4_Anonymous_2022_113898-142_GRG.pdf

2. Response to Request Public 10
   KCA 5.8_Anonymous_2022_113898-148_GRG.pdf
   CA 5.8_Bus_2017_IARC use of oxidative stress as key mode of action_LIT.pdf

3. Response to Request Mammalian Toxicology 24
   KCA 5.5_Anonymous_2022_113898-096_GRG.pdf
   KCA 5.5_Chan and Mahler_1992_NTP technical report_LIT.pdf
   KCA 5.5_Colman_2021_ International Harmonization_LIT.pdf
   KCA 5.5_[redacted]_2022_2107186.000-5839_GRG.pdf
   KCA 5.5_Nolte_2016_Nonproliferative and Proliferative Lesions_LIT.pdf
   KCA 5.5_[redacted]_2022_393-051_GRG.pdf

4. Response to Request Mammalian Toxicology 36
   KCA 5.5_Anonymous_2022_113898-108_GRG.pdf
   KCA 5.9.4_[redacted]_2021_113898-221_GRG.pdf

5. Response to Request Mammalian Toxicology 47
   KCA 5.8.3_Anonymous_2022_113898-119_GRG.pdf
   KCA 9_Anonymous_2022_113898-184_GRG.pdf
   ED summary Table: KCA 5.8.3_Anonymous_2022_113898-119_GRG.xlsx
   Update Appendix E Table: Glyphosate_RAR_Appendix E_eco-tox_STC2022.xlsm

6. Response to Request Mammalian Toxicology 50
   KCA 5.8.1_Anonymous_2022_113898-122_GRG.pdf
   KCA 5.8.1_[redacted]_2022_2177701_GRG.pdf
   KCA 5.8.1_[redacted]_2021_2177900_GRG.pdf
   KCA 5.8.1_[redacted]_2022_2177702_GRG.pdf
   KCA 5.8.1_[redacted]_2022_00050535_draft_report_GRG.pdf
   KCA 4.1.2_[redacted]_2022_LMS-2021-0278_GRG.pdf
   KCA 5.8.1_KCA 4.1.2_[redacted]_2022_00050535_GRG.pdf
   KCA 5.8.1_KCA 4.1.2_[redacted]_2022_00050535_LMS-2021-0278_GRG_sum_analytics.pdf
   KCA 5.8.1_[redacted]_2022_00050535_GRG.pdf

7. Response to Request Mammalian Toxicology 52
   KCA 5.8.1_Anonymous_2022_113898-124_GRG.pdf
   KCA 5.8.1_knoellGermanyGmbH_2022_113898-QSAR-1_GRG.pdf
   KCA 5.8.1_knoellGermanyGmbH_2022_113898-QSAR-1-sup_GRG.pdf

8. Response to Request Mammalian Toxicology 64
   KCA 5.10_Anonymous_2022_113898-136_GRG.pdf

9. Response to Request Mammalian Toxicology 65
   KCA 5.10_Anonymous_2022_113898-137_GRG.pdf
   KCA 5.9.5_Cho_2019_Serial measurement of glyphosate_LIT
   KCA 5.10_[redacted]_2019_MSL0030186_GRG.pdf

10. Response to Request Mammalian Toxicology 68
    KCA 5.5.2_Anonymous_2022_113898-140_GRG.pdf
    KCA 5.5.2_Anonymous_1985_MONGLY00244299_GRG.pdf
    KCA 5.5.2_[redacted]_1985_00150564_GRG.pdf
    KCA 5.5.2_[redacted]_1985_77-2061A_GRG.pdf
    KCA 5.5.2_[redacted]_1985_MONGLY00139358_GRG.pdf

11. Response to Request Mammalian Toxicology 39
    KCA_5.6.1_Anonymous_2022_113898-111_GRG.pdf

12. Response to Request Mammalian Toxicology 40
    KCA_5.6.1_Anonymous_2022_113898-112_GRG.pdf

13. Response to Request Mammalian Toxicology 41
    KCA 5.6.1_Anonymous_2022_113898-113_GRG.pdf
    KCA 5.6.1_Anonymous_2022_22-C007_GRG.pdf

14. Response to Request Mammalian Toxicology 42
    KCA 5.6.2_Anonymous_2022_113898-114_GRG.pdf
    KCA 5.6.2_Anonymous_2022_113898-172_GRG.pdf
    KCA 5.6.2_Anonymous_2022_113898-173_GRG.pdf
    KCA 5.6.2_Anonymous_2022_113898-175_GRG.pdf
    KCA 5.6.2_[redacted]_2022_113898-171_GRG.pdf
    KCA 5.6.2_[redacted]_2022_113898-174_GRG.pdf

15. Response to Request Mammalian Toxicology 43
    KCA 5.6.2.2_Anonymous_2022_113898-115_GRG.pdf

16. Chang *et al*., 2022. Systematic literature review of the epidemiology of glyphosate and neurological outcomes. International Archives of Occupational and Environmental Health. DOI: 10.1007/s00420-022-01878-0. https://doi.org/10.1007/s00420-022-01878-0

17. Moser *et al*., 2022. Glyphosate and neurological outcomes: A systematic literature review of animal studies, Journal of Toxicology and Environmental Health, Part B, 25:4, 162-209. DOI: 10.1080/10937404.2022.2083739.
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