European Commission

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and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

Glyphosate

Volume 3 – B.6.4 (AS)

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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B.6.4.1. In vitro studies

B.6.4.1.1. *In vitro studies – bacterial gene mutation, study 1*

Data point	CA 5.4.1/001
Report author	[redacted]
Report year	2014
Report title	Glyphosate: Reverse Mutation Assay 'Ames Test' using *Salmonella typhimurium* and *Escherichia coli*
Report No	41401854
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) no. 440/2008 Method B13/14 (2008), U.S. EPA OCSPP 870.5100, Japanese MAFF
Deviations from current test guideline OECD 471 (1997)	None
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

Glyphosate (batch: 04062014, purity: 85.79%) was assessed for it's potential to induce gene mutations in bacteria in an Ames test conducted with *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* strain WP2uvrA in the presence and absence of metabolic activation (phenobarbitone / β-naphthoflavone-induced rat liver S9 fraction). Two independent experiments were performed, using the plate-incorporation method (standard plate test, first experiment) and the pre-incubation method (second experiment). Triplicate cultures each were exposed to test item concentrations in the range of 1.5 – 5000 µg/plate (first experiment) and 5 – 5000 µg/plate (second experiment). Negative (untreated), vehicle (DMSO) and positive controls were included in each experiment. After an incubation period of 48 hours, the plates were inspected for a possible reduction in the bacterial background lawn and the number of revertant colonies were counted for each plate.

There was no precipitation observed in any tester strain up to the highest tested concentration, neither in the presence nor absence of S9 mix. Cytotoxicity, evident as a reduction in the bacterial background lawn, was evident in both experiments at 5000 µg/plate for all strains in the absence of metabolic activation. There was no reduction in bacterial growth observed in the presence of S9 mix, however, a reduced frequency in the number of revertant colonies was noted in tester strain TA100 in the first experiment at 5000 µg/plate and in tester strain WP2 uvrA in the second experiment at 5000 µg/plate.

There was no statistically significant increase in the frequency of revertant colonies noted for any of the bacterial strains at any dose level, either with or without S9 mix. The number of revertant colonies for the vehicle control and for negative (untreated) controls were considered acceptable. All of the positive controls induced marked increases in the frequency of revertant colonies, thus confirming the activity of the metabolic activation system and the sensitivity of the test itself.

Based on the experimental findings, the test item is not mutagenic in bacteria with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	Not specified
Description:	White crystalline solid
Lot/Batch #:	04062014
Purity:	85.79%
Stability of test compound:	The stability of the test item under storage conditions (at room temperature in the dark) and in solvent (vehicle) was not specified. All formulations were used within 4 h of preparation and were assumed to be stable for this period.
Solvent (vehicle) used:	Dimethylsulfoxide (DMSO)

2. Control materials:

Negative control:	Untreated controls were included in each experiment.
Solvent (vehicle) control:	Dimethylsulfoxide (DMSO)
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to table below.

Strain	Metabolic activation	Mutagen	Conc. [µg/plate]
S. typhimurium strains			
TA 100	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	3.0
	+S9	2-Aminoanthracene (2-AA)	1.0
TA 1535	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	5.0
	+S9	2-Aminoanthracene (2-AA)	2.0
TA 98	-S9	4-Nitroquinoline-1-oxide (4-NQO)	0.2
	+S9	Benzo[a]pyrene (BP)	5.0
TA 1537	-S9	9-Aminoacrinidine (9AA)	80.0
	+S9	2-Aminoanthracene (2-AA)	2.0
E. coli strain(s)			
WP2 uvrA	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	2.0
	+S9	2-Aminoanthracene (2-AA)	10.0

3. Metabolic activation:

S9 mix was produced from the livers of male rats, which were treated orally with 80 mg/kg bw/day phenobarbitone and 100 mg/kg bw/day β-naphthoflavone for three days. On Day 4, the livers were prepared and S9 homogenate was generated. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
0.2 M Sodium phosphate buffer (pH 7.4)	25.0	mL
1.65 M KCl/0.4 M $MgCl_2$	1.0	mL
NADPH-generating system		
0.1 M Glucose 6-phosphate	2.5	mL
0.1 M NADP	2.0	mL
Sterile distilled water	14.5	mL
S9	5.0	mL

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	Deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		Ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 1538				Histidine auxotrophy (automatically *via* the spontaneous rate)	✓

5. Test concentrations:

Plate incorporation test ± S9 mix (Experiment I):	
Concentrations:	1.5, 5, 15, 50, 150, 500, 1500 and 5000 µg/plate
Tester strains:	TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA
Replicates:	Triplicates
Pre-incubation test ± S9 mix (Experiment II):	
Concentrations:	5, 15, 50, 150, 500, 1500 and 5000 µg/plate
Tester strains:	TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 16 Jul – 31 Aug 2014
Finalisation date: 02 Sep 2014

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution, vehicle or positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were added to 2 mL of molten trace amino acid supplemented medium (1.0 mM histidine + 1.0 mM biotin or 1.0 mM tryptophan). After mixing, the mixture was then overlayed onto a Vogel-Bonner agar plate. Negative (untreated) controls were included in each experiment. The plates were incubated at 37 ± 3 °C for approximately 48 hours and scored for the presence of revertant colonies and thinning of the bacterial background lawn.

3. Pre-incubation test (PIT):
0.1 mL of test solution, vehicle or positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were mixed and pre-incubated for 20 minutes at 37 ± 3 °C while shaking. After pre-incubation, 2.0 mL of molten amino acid supplemented medium (1.0 mM histidine + 1.0 mM biotin or 1.0 mM tryptophan), the mixture was plated onto Vogel-Bonner agar plates. Negative (untreated) controls were included in each experiment. The plates were incubated at 37 ± 3 °C for approximately 48 hours and scored for the presence of revertant colonies and thinning of the bacterial background lawn.

4. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants

- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics

Statistical analysis was performed according to the UKEMS sub-committee on guidelines for mutagenicity testing[1].

6. Acceptance criteria

The test was considered valid if the following criteria were met:

- All bacterial strains must have demonstrated the required characteristics as determined by their respective strain checks.
- All tester strains cultures should exhibit a characteristic number of spontaneous revertants per plate, which is in the range of historical control data.
- All tester strain cultures should be in the range of $0.9 – 9 \times 10^9$ bacteria per mL.
- Positive control chemicals should induce marked increases in the frequency of revertant colonies, both with or without metabolic activation, which are in the range of historical control data.
- There should be a minimum of 4 non-toxic test item concentrations.
- There should be no evidence of excessive contamination.

7. Evaluation criteria

A test item was considered positive for mutagenicity if the following criteria were met:

- There was a dose-related increase in mutant frequency over the dose range tested.
- There was a reproducible increase at one or more concentrations.
- There was a biological relevance against the laboratories historical control ranges.
- The increase fold was greater than two times the concurrent solvent control for any tester strain.

A test item was considered non-mutagenic (negative) in the test system if the above-mentioned criteria were not met.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study as not required by the test guideline

B. CYTOTOXICITY

Cytotoxicity, evident as a visible reduction in the growth of the bacterial background lawn was observed in the first experiment (plate incorporation test) for all tester strains at 5000 µg/plate in the absence of metabolic activation.

There was no reduction in the bacterial background lawn observed in the presence of S9 mix, however, when compared to solvent controls, strain TA 100 exhibited a lower number of revertant colonies at 5000 µg/plate.

In the second experiment (pre-incubation test), cytotoxicity results in the absence of S9 mix were identical to those of the first experiment, showing a reduced bacterial background lawn at 5000 µg/plate for all strains. In the presence of S9 mix, the bacterial growth was not impaired, but a lower frequency of revertant colonies was noted for tester strain WP2 uvrA at 5000 µg/plate.

C. SOLUBILITY

Precipitation of the test item was not observed on the plates at any of the dose levels tested, neither in the presence nor absence of S9 mix.

D. MUTATION ASSAY

There was no statistically significant increase in the frequency of revertant colonies recorded for any of the bacterial strains at any dose of the test item, either with or without metabolic activation. The number of revertant colonies for the vehicle control and for negative (untreated) controls were considered acceptable. In the first and second experiment, two values of the strain TA1535 and two values of strain WP2uvrA were slightly outside (below) of the historical control range, but as the count was only two to three colonies out of range and all other counts were within the range of historical controls, the value was considered acceptable. All of the positive controls

[1] Mahon G.A.T. (1989) Analysis of data from microbial colony assays. In: KIRKLAND D.J. (eds). Statistical Evaluation of Mutagenicity Test Data. Cambridge University Press Report, pp. 26-65

induced marked increases in the frequency of revertant colonies, thus confirming the activity of the metabolic activation system and the sensitivity of the test itself.

Table 6.4.1.1-1: Glyphosate: Reverse Mutation Assay 'Ames Test' using Salmonella typhimurium and Escherichia coli ([redacted] 2014), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Negative control[$]										
mean	72		11		18		19		8	
± SD	± 14.2		± 7.4		± 6.2		± 4.0		± 3.5	
Vehicle control										
DMSO mean	70	82	16	9	18	25	19	22	12	12
± SD	± 5.1	± 9.3	± 8.3	± 3.2	± 6.2	± 3.8	± 5.5	± 9.0	± 4.2	± 3.6
HCD[#] mean	103	101	20	15	28	33	22	26	11	13
± SD	± 14.4	± 15.6	± 4.4	± 3.5	± 6.6	± 7.1	± 5.0	± 5.1	± 3.1	± 3.5
[range]	68 - 147	63 - 153	9 - 37	8 - 29	15 - 47	13 - 54	10 - 42	12 - 43	5 - 26	5 - 23
Test item [µg/plate]										
1.5 mean	71	73	12	13	21	21	14	21	9	10
± SD	± 8.3	± 8.1	± 7.6	± 4.0	± 5.5	± 7.9	± 3.1	± 8.7	± 7.2	± 2.1
5 mean	64	77	12	8	17	20	16	22	14	16
± SD	± 2.3	± 11.5	± 7.5	± 0.6	± 8.7	± 1.0	± 4.0	± 5.7	± 5.0	± 6.1
15 mean	69	90	19	12	21	14	19	20	15	11
± SD	± 6.8	± 7.2	± 5.3	± 2.3	± 4.9	± 2.1	± 6.0	± 8.1	± 5.9	± 3.5
50 mean	72	72	12	14	21	20	12	17	14	12
± SD	± 4.7	± 7.5	± 4.0	± 5.0	± 2.6	± 3.5	± 3.6	± 6.1	± 1.7	± 5.0
150 mean	74	73	16	10	20	22	16	20	8	10
± SD	± 9.5	± 11.0	± 7.2	± 2.9	± 3.5	± 4.9	± 6.1	± 4.2	± 2.3	± 4.0
500 mean	70	87	11	11	17	22	20	18	13	11
± SD	± 3.5	± 7.0	± 4.0	± 6.7	± 6.7	± 9.6	± 4.5	± 4.6	± 0.0	± 4.4
1500 mean	67	67	13	12	19	22	12	14	6	11
± SD	± 11.5	± 4.0	± 4.0	± 0.6	± 0.6	± 2.1	± 3.6	± 5.6	± 1.2	± 7.4
5000 mean	32[S]	48	7[S]	7	11[S]	25	9[S]	16	4[S]	11
± SD	± 8.0	± 14.4	± 2.3	± 2.1	± 3.8	± 4.0	± 7.1	± 1.2	± 1.2	± 2.5
Positive control										
[§] mean	830	1595	866	276	972	349	268	190	407	377
± SD	± 130.0	± 97.9	± 97.5	± 65.7	± 71.2	± 72.5	± 63.1	± 29.3	± 68.5	± 63.5
HCD[#] mean	543	1211	644	250	611	320	207	226	813	286
± SD	± 192.1	± 509.3	± 685.5	± 98.9	± 256.3	± 120.9	± 76.9	± 92.6	± 384.2	± 127.7
[range]	240 - 1429	349 - 3117	91 - 3750	103 - 1153	129 - 1275	101 - 733	102 - 783	84 - 669	113 - 2161	86 - 1238
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[$] Spontaneous mutation rates, untreated plates										

Table 6.4.1.1-1: Glyphosate: Reverse Mutation Assay 'Ames Test' using Salmonella typhimurium and Escherichia coli ([redacted], 2014), first experiment										
Experiment 1: Standard plate test (SPT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[#] Historical control data from 2013, HCD of untreated and vehicle controls are combined, n = 6-1655 plates (combined vehicle and untreated control values), n = 6-861 plates (positive control values). It is unclear whether data are from standard plate tests, pre-incubation test, or from a combined dataset.										
[S] Sparse bacterial background lawn										

Table 6.4.1.1-2: Glyphosate: Reverse Mutation Assay 'Ames Test' using *Salmonella typhimurium* and *Escherichia coli* ([redacted], 2014), second experiment										
Experiment 2: Pre-incubation Test (PIT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Negative control[$]										
mean	100		15		19		24		14	
± SD	± 10.6		± 6.2		± 4.5		± 7.0		± 2.3	
Vehicle control										
DMSO mean	90	87	16	13	19	21	21	23	17	8
± SD	± 4.6	± 18.4	± 3.5	± 2.1	± 1.7	± 5.1	± 4.0	± 5.3	± 3.2	± 3.1
HCD[#] mean	103	101	20	15	28	33	22	26	11	13
± SD	± 14.4	± 15.6	± 4.4	± 3.5	± 6.6	± 7.1	± 5.0	± 5.1	± 3.1	± 3.5
[range]	68 - 147	63 - 153	9 - 37	8 - 29	15 - 47	13 - 54	10 - 42	12 - 43	5 - 26	5 - 23
Test item										
5 mean	91	76	9	16	17	21	22	18	13	9
± SD	± 4.6	± 15.0	± 1.7	± 7.5	± 2.5	± 4.4	± 10.3	± 1.7	± 4.7	± 2.1
15 mean	92	88	13	13	20	22	25	27	12	8
± SD	± 8.7	± 9.2	± 6.5	± 4.4	± 3.1	± 1.7	± 3.8	± 6.2	± 3.1	± 0.6
50 mean	99	72	13	8	22	20	18	22	14	13
± SD	± 7.0	± 12.4	± 6.8	± 1.2	± 3.1	± 5.0	± 9.5	± 9.8	± 4.9	± 6.6
150 mean	83	91	10	12	22	18	22	26	12	14
± SD	± 7.0	± 10.8	± 2.3	± 0.6	± 10.5	± 4.2	± 6.7	± 6.0	± 4.0	± 1.2
500 mean	62	79	11	14	17	21	21	17	12	13
± SD	± 2.1	± 4.6	± 4.0	± 1.7	± 1.5	± 2.0	± 7.4	± 1.5	± 0.0	± 8.4
1500 mean	70	79	11	8	16	14	20	19	8	7
± SD	± 15.9	± 3.1	± 2.9	± 0.6	± 5.0	± 3.1	± 1.0	± 2.1	± 0.6	± 2.9
5000 mean	65[S]	94	6[S]	9	12[S]	11	16[S]	20	6[S]	9
± SD	± 17.3	± 9.1	± 1.2	± 0.6	± 0.0	± 4.6	± 3.6	± 7.5	± 1.7	± 3.8
Positive control										
[§] mean	1278	1279	2080	228	1183	298	407	163	977	545
± SD	± 223.2	± 79.3	± 464.1	± 22.0	± 176.3	± 27.6	± 31.8	± 5.0	± 166.9	± 43.9
HCD[#] mean	543	1211	644	250	611	320	207	226	813	286

± SD	± 192.1	± 509.3	± 685.5	± 98.9	± 256.3	± 120.9	± 76.9	± 92.6	± 384.2	± 127.7
[range]	240 - 1429	349 - 3117	91 - 3750	103 - 1153	129 - 1275	101 - 733	102 - 783	84 - 669	113 - 2161	86 - 1238
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[$] Spontaneous mutation rates, untreated plates										
[#] Historical control data from 2013, HCD of untreated and vehicle controls the values were combined, n = 6-1655 plates (combined vehicle and untreated control values), n = 6-861 plates (positive control values). It is unclear whether data are from standard plate tests, pre-incubation test, or from a combined dataset.										
[S] Sparse bacterial background lawn										

III. CONCLUSION

Under the conditions of the present study, the test item is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA100, TA98, TA1535 and TA1537 and *E. coli* WP2uvrA) in the presence and absence of metabolic activation.

The study was performed according to OECD guideline 471 (1997) and compliant with GLP. It is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2uvrA) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.2. *In vitro studies – bacterial gene mutation, study 2*

Data point	CA 5.4.1/002
Report author	[redacted]
Report year	2012
Report title	Reverse Mutation Assay using Bacteria (*Salmonella typhimurium*) with Glyphosate tech.
Report No	126159
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) No. 440/2008 B.13/14 (2008), US EPA OPPTS 870.5100 (1998)
Deviations from current test guideline OECD 471 (1997)	None
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

Glyphosate technical (batch: 20110107-2, purity: 97%) was assessed for its potential to induce gene mutations in bacteria in an Ames test conducted in *S. typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537. All strains were exposed, to the test item, solvent (DMSO), negative (water) and appropriate positive controls in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for at least 48 h.

Concentrations were selected based on the results of a preliminary cytotoxicity test, in which the test item induced cytotoxicity in strain TA 100 at ≥ 2500 µg/plate in the presence of metabolic activation. Test item concentrations for the main mutagenicity study ranged from 10 to 5000 µg/plate. Two independent experiments were performed,

using the plate incorporation method (standard plate test, experiment 1) and the pre-incubation method (experiment 2).

There was no precipitation observed up to the highest concentration tested, neither in the presence, nor in the absence of metabolic activation. Cytotoxicity was observed in the first experiment for strain TA98 at 5000 µg/plate in the presence of S9 mix, for strain TA 100 at ≥ 2500 µg/plate in the presence and absence of S9 mix and in strains TA 1535 and TA 102 at 5000 µg/plate in the absence of S9 mix.

In the second experiment, cytotoxicity was observed in strains TA 98 and TA 100 at 5000 µg/plate in the absence of S9 mix, in strain TA 1537 at ≥ 2500 µg/plate in the absence of S9 mix and in strain TA102 at 5000 µg/plate in the presence and absence of S9 mix.

Upon treatment with glyphosate technical, there was no statistically significant or biologically relevant increase in the number of revertant colonies in any of the five tester strains in any experiment up to the highest tested concentration, neither in the presence, nor in the absence of S9 mix.
The number of revertant colonies in the solvent, negative and positive control was within the range of the laboratories historical control data, demonstrating the functionality of the S9 mix and the validity of the test system.

Under the experimental conditions, glyphosate technical was considered negative for gene mutation in bacteria with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate Tech. (N-phosphonomethyl)glycine

Identification:	Not specified
Description:	Not specified
Lot/Batch #:	20110107-2
Purity:	97%
Stability of test compound:	The stability of the test item under storage conditions (at room temperature, protected from light) was guaranteed until 01 Feb 2013. The stability of the test item in solvent (vehicle) was not specified.
Solvent (vehicle) used:	Dimethylsulfoxide (DMSO)

2. Control materials:

Negative control:	Distilled water
Solvent (vehicle) control:	Dimethylsulfoxide (DMSO)
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)[1]	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)[1]	DMSO	2.5
TA 98	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)[1]	DMSO	2.5
TA 1537	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	40.0
	+S9	2-Aminoanthracene (2-AA)[1]	DMSO	2.5
TA 102	-S9	Methylmethanesulfonate (MMS)	Water	1.0 µL/plate
	+S9	2-Aminoanthracene (2-AA)[1]	DMSO	10.0

[1] The functionality of the S9 mix batch used was checked with benzo(a)pyrene and showed the expected results

3. Metabolic activation:

S9 mix was prepared by [redacted], Experiment 1) and purchased from [redacted], Experiment 2). S9 mix from [redacted] was obtained from the livers of male Wistar rats, that received phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) by oral treatment for three consecutive days. S9 mix from [redacted] was produced from male Sprague-Dawley rats, which were induced with phenobarbital / β-naphthoflavone.
Prior to each experiment, co-factor was added to the S9 mix containing the following components:

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	5	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102	✓				
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

1. Preliminary cytotoxicity assay:

Plate incorporation test ± S9 mix:	
Concentrations:	3.16, 10, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 98 and TA 100
Replicates:	Triplicates

2. Mutation assays:

Plate incorporation test ± S9 mix:	
Concentrations:	10, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates:	Triplicates
Pre-incubation test ± S9 mix:	
Concentrations:	10, 31.6, 100, 316, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

A. Dates of experimental work: 13 Nov - 10 Dec 2012
Finalisation date: 17 Dec 2012

B. Standard plate test (plate-incorporation test, SPT):

An aliquot of 0.1 mL test solution, negative control (water), solvent control (DMSO) or positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten overlay agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin). All components were mixed in a test tube and poured onto minimal agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C upside down for at least 48 h in the dark, the background bacterial lawn was examined and the bacterial colonies (his^+ revertants) were counted.

C. Pre-incubation test (PIT):

0.1 mL of test solution, negative control (water), solvent control (DMSO) or positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were pre-incubated at 37 °C for 60 minutes, followed by addition of 2 mL molten overlay agar. Each concentration and the controls was tested in triplicates. The mixture was poured onto the surface of a minimal agar plate and after solidification, the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ revertants) was counted.

D. Cytotoxicity

Toxicity was detected by a

- reduction in the number of spontaneous revertants (approx. 50 % reduced in relation to the solvent control).
- clearing or diminution of the background lawn (= reduced background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

E. Statistics

Results were judged without statistical analysis.

F. Acceptance criteria

The test was valid if the following criteria were met:

- The bacteria demonstrated their typical responses to ampicillin (TA 98, TA 100 and TA 102).
- The control plates with and without S9 mix were within the ranges of the laboratory's historical data.
- Corresponding background growth on negative control, solvent control and test plates was observed.
- The positive controls showed a distinct enhancement of revertant rates over the control plate.

G. Evaluation criteria

A test item was considered as mutagenic if the following criteria were met:

- A clear and dose-related increase in the number of revertants occurred and/or
- A biologically relevant positive response for at least one of the dose groups occurred in at least one tester strain with or without metabolic activation. A biologically relevant increase was obtained when the number of revertants was at least two-fold when compared to solvent controls for testers strains TA 98, TA 100 and TA 102 and at least three-fold when compared to solvent controls for tester strains TA 1535 and TA 1537.

A test item was considered non-mutagenic if it produced neither a dose-related increase in the number of revertants nor a reproducible biologically relevant positive response at any of the dose levels.

1. RESULTS AND DISCUSSION

I. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study as not required by the test guideline.

II. CYTOTOXICITY

In the preliminary toxicity test, cytotoxicity was observed in strain TA 100 at ≥ 2500 µg/plate in the presence of metabolic activation.

In the main mutagenicity assay, cytotoxicity was observed in the first experiment (standard plate test = plate-incorporation method) for strain TA98 at 5000 µg/plate in the presence of S9 mix, for strain TA 100 at ≥ 2500 µg/plate in the presence and absence of S9 mix and in strains TA 1535 and TA 102 at 5000 µg/plate in the absence of S9 mix.

In the second experiment (pre-incubation test), cytotoxicity was observed in strains TA 98 and TA 100 at 5000 µg/plate in the absence of S9 mix, in strain TA 1537 at ≥ 2500 µg/plate in the absence of S9 mix and in strain TA 102 at 5000 µg/plate in the presence and absence of S9 mix.

The reduction in the number of revertant colonies in strain TA 1537 at 100 µg/plate in the absence of S9 mix was regarded as not biologically relevant, due to a lack of a dose-response relationship.

III. SOLUBILITY

There was no precipitation of the test substance observed up to the highest dose tested in any of the experiments, neither in the presence nor absence of S9 mix.

IV. MUTATION ASSAY

There was no statistically significant or biologically relevant increase in the number of his^+ revertant colonies in any of the five tester strains in any of the two experiments up to the highest tested concentration, neither in the presence, nor in the absence of S9 mix.

The number of revertant colonies in the solvent, negative and positive control was within the range of the laboratories historical control data. The positive controls induced a marked increase in the number of revertant colonies, demonstrating the functionality of the S9 mix and the validity of the test system.

Table B.6.4.1.2-1: Reverse Mutation Assay using Bacteria (Salmonella typhimurium) with Glyphosate Tech ([redacted], 2012), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	22	26	88	115	23	14	6	9	232	333
± SD	± 3.0	± 8.1	± 6.4	± 13.4	± 3.1	± 2.6	± 2.9	± 3.2	± 3.5	± 37.3
DMSO mean	15	27	81	89	18	11	6	8	202	287
± SD	± 4.7	± 2.6	± 6.6	± 11.8	± 4.0	± 5.6	± 2.6	± 2.1	± 10.7	± 18.0
HCD[#] mean	23.8	30.6	113.3	114.8	10.0	8.7	8.8	9.1	252.1	288.0
± SD	± 5.8	± 5.1	± 16.2	± 16.2	± 2.9	± 2.3	± 2.9	± 3.0	± 46.6	± 58.0
[range]	16 - 46	18 - 53	77 - 174	79 - 162	5 - 27	5 - 26	5 - 28	5 - 32	164 - 399	169 - 467
Test item [µg/plate]										
10 mean	11	23	85	83	19	18	6	7	225	338
± SD	± 1.0	± 3.1	± 13.0	± 4.6	± 3.8	± 3.1	± 3.5	± 3.1	± 20.4	± 16.0
31.6 mean	20	25	77	90	21	11	7	6	219	314
± SD	± 4.0	± 1.0	± 5.6	± 2.0	± 2.9	± 2.0	± 2.1	± 1.2	± 37.9	± 13.8
100 mean	14	21	86	93	18	9	4	4	225	288
± SD	± 1.5	± 0.6	± 8.2	± 9.0	± 2.1	± 1.5	± 2.9	± 1.2	± 14.2	± 8.7
316 mean	15	24	75	103	15	11	7	7	197	251
± SD	± 1.5	± 4.4	± 7.6	± 15.3	± 7.9	± 3.5	± 0.6	± 2.1	± 3.6	± 13.8
1000 mean	14	21	73	88	16	11	6	6	187	254
± SD	± 2.1	± 3.5	± 7.4	± 7.9	± 3.6	± 3.2	± 2.3	± 2.0	± 8.5	± 28.4
2500 mean	10	20	49[B]	85[B]	18	14	6	7	203	331
± SD	± 1.0	± 6.4	± 15.0	± 19.1	± 2.1	± 4.6	± 2.6	± 3.0	± 3.2	± 25.2
5000 mean	12	15	19[B]	53[B]	9[B]	12	4	6	77	255
± SD	± 5.5	± 7.1	± 8.5	± 15.3	± 2.3	± 4.4	± 3.2	± 1.0	± 42.5	± 9.0
Positive control										
[§] mean	299	1906	390	1533	1423	203	52	130	1338	769
± SD	± 25.5	± 219.0	± 73.4	± 369.8	± 27.1	± 13.7	± 8.5	± 27.6	± 263.3	± 66.4
HCD[#] mean	491.8	2283.1	933.9	1759.0	1031.9	115.7	125.2	237.6	1516.9	1043.6
± SD	± 154.9	± 651.4	± 263.7	± 508.4	± 287.1	± 59.4	± 30.6	± 91.7	± 335.0	± 305.9
[range]	205 - 2613	313 - 3587	279 - 1876	462 - 3204	67 - 1850	27 - 732	34 - 275	32 - 474	391 - 2902	371 - 2422

[§] Information on respective positive control is reported in Material and Method section I.A.2

Table B.6.4.1.2-1: Reverse Mutation Assay using Bacteria (Salmonella typhimurium) with Glyphosate Tech ([redacted], 2012), first experiment										
Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[#] Historical control data generated from 2009 – 2011 (n = 631-1058 for the negative control; n = 469-1046 for the positive control). It is unclear whether data are from standard plate tests, pre-incubation test, or from a combined dataset.										
[B] Background lawn reduced										

Table B.6.4.1.2-2: Reverse Mutation Assay using Bacteria (Salmonella typhimurium) with Glyphosate Tech [redacted], 2012), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	21	29	110	103	14	12	6	7	215	215
± SD	± 2.0	± 9.2	± 3.5	± 20.7	± 3.1	± 6.2	± 3.2	± 1.5	± 15.8	± 4.4
DMSO mean	20	23	104	94	11	12	8	5	171	184
± SD	± 7.5	± 3.6	± 6.5	± 7.4	± 4.6	± 4.5	± 0.7	± 2.1	± 17.7	± 11.5
HCD[#] mean	23.8	30.6	113.3	114.8	10.0	8.7	8.8	9.1	252.1	288.0
± SD	± 5.8	± 5.1	± 16.2	± 16.2	± 2.9	± 2.3	± 2.9	± 3.0	± 46.6	± 58.0
[range]	16 - 46	18 - 53	77 - 174	79 - 162	5 - 27	5 - 26	5 - 28	5 - 32	164 - 399	169 - 467
Test item										
10 mean	23	30	119	98	17	13	10	4	163	233
± SD	± 8.0	± 7.5	± 18.6	± 16.6	± 2.5	± 2.5	± 3.2	± 2.0	± 10.6	± 7.0
31.6 mean	24	29	114	115	17	12	6	5	167	233
± SD	± 1.5	± 7.2	± 9.1	± 8.4	± 3.6	± 3.0	± 1.0	± 1.7	± 15.9	± 8.7
100 mean	18	26	104	115	19	8	3	5	147	210
± SD	± 5.5	± 3.2	± 4.7	± 6.7	± 0.0	± 2.1	± 2.1	± 2.0	± 12.4	± 14.0
316 mean	20	22	113	102	15	11	4	6	155	210
± SD	± 3.5	± 5.3	± 4.5	± 10.4	± 1.5	± 4.6	± 2.3	± 1.0	± 7.8	± 10.0
1000 mean	25	32	131	108	13	13	6	3	170	230
± SD	± 5.6	± 9.5	± 33.3	± 14.2	± 2.9	± 0.6	± 5.3	± 2.1	± 10.6	± 21.8
2500 mean	22	26	114	89	15	11	4	11	178	229
± SD	± 5.7	± 10.8	± 11.0	± 8.5	± 5.3	± 4.7	± 2.6	± 4.6	± 3.5	± 24.6
5000 mean	7	26	21[B]	100	8	12	2	6	37	61
± SD	± 3.2	± 3.8	± 16.6	± 2.1	± 2.1	± 2.5	± 0.6	± 4.0	± 16.5	± 25.3
Positive control										
[§] mean	552	1958	1102	2002	1220	197	117	151	1972	530
± SD	± 119.9	± 674.0	± 74.8	± 360.7	± 114.0	± 28.3	± 25.0	± 21.8	± 201.8	± 63.0

Table B.6.4.1.2-2: Reverse Mutation Assay using Bacteria (Salmonella typhimurium) with Glyphosate Tech ([redacted], 2012), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
HCD[#] mean	491.8	2283.1	933.9	1759.0	1031.9	115.7	125.2	237.6	1516.9	1043.6
± SD	± 154.9	± 651.4	± 263.7	± 508.4	± 287.1	± 59.4	± 30.6	± 91.7	± 335.0	± 305.9
[range]	205 - 2613	313 - 3587	279 - 1876	462 - 3204	67 - 1850	27 - 732	34 - 275	32 - 474	391 - 2902	371 - 2422
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated from 2009 – 2011 (n = 631-1058 for the negative control; n = 469-1046 for the positive control). It is unclear whether data are from standard plate tests, pre-incubation test, or from a combined dataset.										
[B] Background lawn reduced										

2. CONCLUSION

In conclusion, based on the experimental findings, glyphosate tech. did not induce gene mutations by base pair exchanges or frameshifts in the genome of the five tested *S. typhimurium* strains. Therefore, the test item was considered non-mutagenic in the bacterial reverse mutation test (Ames test).

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and under GLP conditions. There were no deviations when compared to the guideline. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate technical was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537, and TA 102) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.3. *In vitro studies – bacterial gene mutation, study 3*

Data point	CA 5.4.1/003
Report author	[redacted]
Report year	2010
Report title	Mutagenicity study of Glyphosate TC in the *Salmonella typhimurium* Reverse Mutation Assay (*in vitro*)
Report No	24880
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) No. 440/2008 B.13/14 (2008), US EPA OPPTS 870.5100 (1998)
Deviations from current test guideline OECD 471 (1997)	Historical control data for the positive controls were not included in the study report. Furthermore, information in the study report on the HCD for the negative controls is limited.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** Some concerns were raised regarding the work conducted at this specific testing facility. After consultation with the responsible GLP

	monitoring authority, no GLP not in compliance (nic) reports on studies with glyphosate by this testing facility are available or known. As some deviations regarding the historical controls (see above) were identified, the study is considered acceptable but with restrictions.

Glyphosate technical (batch: 2009051501, purity 95.23%) was investigated for its potential to induce gene mutation in bacteria in an Ames test. In the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction), *S. typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 were exposed to the test item, vehicle (aqua ad iniectabilia) and positive controls for 48 – 72 hours at 37 °C.

Based on the results of a preliminary cytotoxicity test conducted in strain TA 100 in the absence of metabolic activation, test item concentrations in the main mutagenicity test ranged from 31.6 to 3160 µg/plate. Two independent experiments were performed, using the standard plate test (plate-incorporation method) and the pre-incubation test. After 48 to 72 hours of incubation, the number of revertant colonies was counted and the bacterial background lawn was examined.

Precipitation of the test item was observed in the preliminary toxicity study at 5000 µg/plate, but at no concentration used in the main mutagenicity test. For all tester strains in the presence and absence of metabolic activation, cytotoxicity was observed at 3160 µg/plate.

There was no increase in the number of revertant colonies when compared to control counts observed in any of the five tester strains following treatment with glyphosate technical tested up to 3160 µg/plate, neither in the presence nor in the absence of metabolic activation.

In both experiments, the number of revertants induced by the vehicle control was within the range of the historical control data for each strain. A distinct increase in the number of revertant colonies was observed for the positive controls, demonstrating the functionality of the S9 mix and the sensitivity of the test system.

Based on the result of the present study and under the experimental conditions of the test, glyphosate technical is negative for mutagenicity in bacteria in the Ames standard plate and pre-incubation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate TC

Identification:	37/206/09
Description:	White powder
Lot/Batch #:	2009051501
Purity:	95.23%
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until the mentioned expiry date 15 May 2011. The stability of the test item in vehicle was not specified in the study report.
Solvent (vehicle) used:	Aqua ad iniectabilia

2. Control materials:

Negative control:	Sterility controls were not included in the present study
Solvent (vehicle) control:	Aqua ad iniectabilia
Solvent (vehicle) /final concentration:	1 mL per plate
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide	Water	10.0
	+S9	Cyclophosphamide	Water	1500.0
TA 1535	-S9	Sodium azide	Water	10.0
	+S9	Cyclophosphamide	Water	1500.0
TA 98	-S9	2-Nitrofluorene	DMSO	10
	+S9	2-Aminoanthracene	DMSO	2.0
TA 1537	-S9	9-Aminoacridine	Ethanol	100.0
	+S9	2-Aminoanthracene	DMSO	2.0
TA 102	-S9	Methylmethane sulfonate	DMSO	1300.0
	+S9	2-Aminoanthracene	DMSO	2.0

3. Metabolic activation:

S9 mix was obtained from the livers of 20 – 30 rats treated with Aroclor 1254. The pooled fraction exhibited a protein content of 26.6 mg/mL and a cytochrome P450 level of 0.21 nmol/protein. Aliquots of the S9 mix were thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100.0	mM
KCl	33.0	mM
NADPH-generating system		
Glucose 6-phosphate	5.4	mM
NADP	4.1	mM
$MgCl_2$	8.0	mM
S9	5.0	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E. coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB genes)	✓
TA 1537	✓				
TA 102	✓				
TA 1538				Histidine auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

1. Preliminary cytotoxicity assay

Plate incorporation test - S9 mix:	
Concentrations:	0.316, 1.0, 3.16, 10.0, 31.6, 100, 316, 1000, 3160 and 5000 µg/plate
Tester strain:	TA 100
Replicates:	Duplicates

2. Main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000 and 3160 µg/plate
Tester strains:	TA 98, TA 100, TA 102, TA 1535 and TA 1537
Replicates:	Triplicates
Pre-incubation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000 and 3160 µg/plate
Tester strains:	TA 98, TA 100, TA 102, TA 1535 and TA 1537
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

A. Dates of experimental work: 15 Oct – 23 Nov 2009
Finalisation date: 25 Jan 2010

B. Standard plate test (plate-incorporation test, SPT):
An aliquot of 1 mL test solution or vehicle or 0.1 mL positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 phosphate buffer (in tests without metabolic activation) were added to 2.0 mL of molten top agar (supplemented with 0.5 mM L-histidine + 0.5 mM biotin). The test components were mixed by vortexing at low speed, poured onto coded minimal glucose agar plates and the plates were quickly tilted and rotated for homogenous distribution. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for 48 to 72 hours at 37 °C in the dark. After incubation, the number of revertant colonies was counted and the presence of bacterial background lawn was confirmed.

C. Pre-incubation test (PIT):
1 mL of test solution or vehicle or 0.1 mL of positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix phosphate buffer (in tests without metabolic activation) were pre-incubated for 20 minutes at 37 °C using a shaker.
Afterwards, 2.0 mL of molten top agar was added to each test tube and the mixture was poured on coded minimal glucose agar plates. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for 48 to 72 hours at 37 °C in the dark. After incubation, the number of revertant colonies was counted and the presence of bacterial background lawn was examined.

3. Cytotoxicity
Toxicity was detected by

- a reduction in the number of spontaneous revertants (below 50 % when compared to solvent controls)
- a clearing or diminution of the background lawn (= reduced his^- background growth)
- the degree of survival of the treated cultures

and recorded for all test groups both with and without S9 mix in all experiments.

4. Statistics
Statistical significance for an increased number of revertants compared to the solvent control was determined using the Mann-Whitney U-test. A concentration-related effect was identified using the Spearman's rank correlation coefficient.

5. Acceptance criteria
Acceptance criteria were not defined in the study report.

6. Evaluation criteria
A test item was considered to show a positive response if the following criteria were met:

- The number of revertant colonies was statistically significantly increased compared to the solvent control to at least 2-fold of the solvent control for TA 98, TA 100 and TA 102 and 3-fold of the solvent control for TA 1535 and TA 1537 in both experiments.
- There was a statistically significant concentration-related effect.
- Positive results were reproducible and the histidine independence of the revertants was confirmed by streaking random samples on histidine-free agar plates

II. RESULTS AND DISCUSSION

D. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

E. CYTOTOXICITY
In the preliminary toxicity test, cytotoxicity was observed in tester strain TA 100 at concentrations of 3160 µg/plate and above in the absence of metabolic activation. Hence, 3160 µg/plate was chosen as top concentration for the main mutagenicity study.

In the main mutation assay, cytotoxicity, indicated by scarce background lawn and a reduction in the number of revertants, was noted at the top concentration of 3160 µg/plate for all tester strains in both experiments (plate-incorporation and pre-incubation test), both in the presence and absence of metabolic activation.

F. SOLUBILITY

Precipitation of the tests item was observed in the preliminary toxicity test in tester strain TA 100 at the top concentration of 5000 µg/plate without metabolic activation. There was no precipitation in the main mutagenicity study.

G. MUTATION ASSAY

There was no increase in the number of revertant colonies when compared to control counts observed in any of the five tester strains following treatment with glyphosate technical tested up to 3160 µg/plate, neither in the presence nor in the absence of metabolic activation.

In both experiments, the number of revertants induced by the vehicle control was within the range of the historical control data for each strain. A distinct increase in the number of revertant colonies was observed for the positive controls, demonstrating the functionality of the S9 mix and the sensitivity of the test system.

Table B.6.4.1.3-1: Mutagenicity study of Glyphosate TC in the *Salmonella typhimurium* Reverse Mutation Assay (*in vitro*) ([redacted], 2010), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Aqua ad iniectabilia mean	30.7	31.0	160.0	141.7	270.3	283.3	21.7	19.7	5.0	7.0
± *SD*	*± 3.2*	*± 8.7*	*± 19.3*	*± 15.9*	*± 3.2*	*± 20.0*	*± 2.5*	*± 2.1*	*± 1.0*	*± 1.0*
HCD[#] range	*20 - 60*		*100 - 200*		*240 - 320*		*10 - 35*		*3 - 20*	
Test item [µg/plate]										
31.6 mean	26.3	29.3	151.3	166.0	261.3	267.7	15.7	15.7	4.7	7.0
± *SD*	*± 5.5*	*± 4.2*	*± 25.7*	*± 17.5*	*± 8.1*	*± 12.5*	*± 1.5*	*± 2.1*	*± 1.5*	*± 1.0*
100 mean	27.7	29.3	148.0	148.3	275.7	274.3	17.3	15.7	4.3	5.3
± *SD*	*± 6.4*	*± 5.8*	*± 7.5*	*± 7.1*	*± 9.3*	*± 14.5*	*± 2.1*	*± 3.5*	*± 1.2*	*± 0.6*
316 mean	25.0	24.3	142.7	148.7	271.3	281.0	17.0	18.3	4.0	4.0
± *SD*	*± 3.6*	*± 5.1*	*± 14.2*	*± 14.4*	*± 8.0*	*± 7.0*	*± 1.7*	*± 3.5*	*± 1.0*	*± 1.0*
1000 mean	31.0	21.3	136.3	136.0	257.0	271.0	19.0	16.0	2.7	5.0
± *SD*	*± 4.6*	*± 3.2*	*± 21.2*	*± 18.5*	*± 17.3*	*± 11.8*	*± 3.6*	*± 1.0*	*± 0.6*	*± 1.7*
3160 mean	13.3[$]	8.3[$]	65.3[$]	106.0[$]	226.3[$]	265.7[$]	9.7[$]	13.0[$]	2.3[$]	2.0[$]
± *SD*	*± 1.5*	*± 2.5*	*± 11.8*	*± 4.0*	*± 6.0*	*± 1.5*	*± 2.1*	*± 1.7*	*± 1.5*	*± 1.0*
Positive control										
[§] mean	352.0	333.3	894.7	909.3	1038.3	1034.0	247.7	243.7	210.3	212.0
± *SD*	*± 52.0*	*± 8.3*	*± 87.9*	*± 69.3*	*± 7.8*	*± 11.1*	*± 64.3*	*± 22.2*	*± 9.5*	*± 5.6*

[§] Information on respective positive control is reported in Material and Method section I.A.2

[#] Historical control data generated in the laboratory of the testing facility (no further details available in the study report).

[$] Cytotoxicity indicated by scarce background lawn

Table B.6.4.1.3-2: Mutagenicity study of Glyphosate TC in the *Salmonella typhimurium* Reverse Mutation Assay (in vitro) ([redacted], 2010), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Aqua ad iniectabilia mean	35.0	36.0	171.0	136.7	282.0	294.7	24.0	19.3	8.0	9.0
± SD	± 4.0	± 3.6	± 24.0	± 8.0	± 24.2	± 26.1	± 3.6	± 3.1	± 1.7	± 1.0
HCD[#] range	20 - 60		100 - 200		240 - 320		10 - 35		3 - 20	
Test item [µg/plate]										
31.6 mean	34.0	35.0	139.7	132.0	275.0	283.7	16.7	20.7	5.3	8.3
± SD	± 3.5	± 3.0	4.6	± 14.0	± 8.9	± 9.0	± 1.5	± 1.5	± 1.5	± 1.5
100 mean	30.7	35.7	151.7	129.3	276.0	293.7	20.3	17.0	5.7	7.3
± SD	± 7.6	± 2.9	± 24.6	± 21.4	± 10.1	± 8.4	± 2.1	± 2.6	± 1.5	± 1.5
316 mean	30.0	32.7	169.7	120.3	275.0	285.0	19.7	19.0	6.0	7.7
± SD	± 5.3	± 4.0	± 17.2	± 8.3	± 12.5	± 7.0	± 2.1	± 2.6	± 1.0	± 0.6
1000 mean	30.7	29.3	148.3	109.3	248.3	250.3	20.3	18.0	5.0	6.3
± SD	± 4.5	± 3.5	± 11.2	± 2.3	± 3.1	± 2.1	± 3.2	± 2.6	± 1.7	± 1.5
3160 mean	5.3[$]	6.3[$]	50.7[$]	38.0[$]	75.3[$]	74.7[$]	8.3[$]	8.0[$]	1.3[$]	1.7[$]
± SD	± 2.5	± 1.5	± 5.1	± 3.0	± 14.5	± 10.0	± 1.5	± 2.0	± 0.6	± 0.6
Positive control										
[§] mean	397.7	427.0	934.0	943.3	1040.7	1078.3	303.3	362.3	243.7	235.3
± SD	± 24.5	± 28.5	± 72.0	± 51.2	± 28.9	± 28.6	± 29.7	± 40.1	± 21.2	± 12.0

[§] Information on respective positive control is reported in Material and Method section I.A.2

[#] Historical control data generated in the laboratory of the testing facility (no further details available in the study report).

[$] Cytotoxicity indicated by scarce background lawn

III. CONCLUSION

According to the results and under the conditions of the present study, glyphosate technical is not mutagenic in the Ames standard plate and pre-incubation test with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537) with and without metabolic activation.
The study was performed according to OECD guideline 471 (1997) and under GLP conditions. Deviations from the guideline were considered to be of minor degree that do not hinder data evaluation. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate TG was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537, and TA 102) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.4. *In vitro studies – bacterial gene mutation, study 4*

Data point	CA 5.4.1/004
Report author	[redacted]
Report year	2010
Report title	*Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine
Report No	1332300
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) No. 440/2008 B13/14 (2008)
Deviations from current test guideline OECD 471 (1997)	2-Aminoanthracene was used as sole positive control in the presence of metabolic activation, but the functionality of the S9 batch was routinely checked with benzo(a)pyrene according to the study author. Furthermore, the positive controls showed marked increases in the number of revertants. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

Glyphosate technical (batch: 2009051501, purity 97.16%) spiked with glyphosine was investigated with regard to its potential to induce gene mutation in bacteria in an Ames test. *S. typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strain WP2 uvrA were exposed to the test item in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for at least 48 hours at 37 °C. Vehicle (deionized water), untreated and positive controls were included in each experiment.

A pre-experiment was conducted to identify cytotoxic concentrations of the test item. In a standard plate test (plate incorporation method), test item concentrations in the range of 3 to 5000 µg/plate did not show any cytotoxic effects in the presence and absence of metabolic activation. The pre-experiment was designated experiment I of the main mutation assay and analysed for the number of revertant colonies. A second main mutation assay was performed using the preincubation method and concentrations in the same concentration range. In both experiments, all conditions were tested in triplicates. After at least 48 hours of incubation, the mean number of revertant colonies was counted for each plate.

Precipitation of the test substance was observed for all tester strains in the second experiment only (preincubation method) at ≥ 2500 µg/mL in the presence of metabolic activation. In none of the experiments, cytotoxicity was observed.

There was no substantial increase in the number of his^+ or trp^+ revertant colonies observed in any of the five tester strains at any dose level, neither in the presence nor in the absence of metabolic activation.
The number of revertants induced by the vehicle control was within the range of the historical control data for each strain, thus demonstrating an acceptable experimental performance. Appropriate positive control compounds showed a distinct increase in the number of revertant colonies, confirming the activity of the S9 mix and the validity of the test system.

Based on the results of the present study and under the experimental conditions of the test, glyphosate technical spiked with glyphosine did not cause any genotoxic effect in the Ames standard plate and pre-incubation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical (5000 mg/L), containing glyphosine (32 mg/L)

Identification: S 11113 11

Description: An aqueous solution of glyphosate technical grade active ingredient (purity 97.16% w/w), containing 0.63% (w/w) glyphosine in the technical grade active ingredient.

Lot/Batch #: 2009051501 (glyphosate TC); 1438405 (glyphosine)

Purity: 97.16%

Stability of test compound:	The stability of the test item under storage conditions (at room temperature, light protected) was guaranteed for two weeks from the date of preparation. The stability of the test item in vehicle was not specified in the study report.
Solvent (vehicle) used:	Deionised water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Deionised water
Solvent (vehicle)/final concentration:	0.1 mL per plate
Positive controls:	Please refer to the table below.

Strain	**Metabolic activation**	**Mutagen**	**Solvent**	**Conc. [µg/plate]**
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 98	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1537	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	50.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
E. coli strain				
WP2 uvrA	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0

* The functionality of the S9 mix batch used was routinely checked with benzo(a)pyrene and showed the expected results.

3. Metabolic activation:

S9 mix was obtained from the livers of 8 – 12 weeks old male Wistar rats, weighing approx. 220 - 320 g. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and oral administration of β-naphthoflavone (80 mg/kg bw) each on three consecutive days. The livers were prepared 24 h after the last treatment. Aliquots of the S9 mix were thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E. coli***			
TA 98	✓	WP2 *uvr*A	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 *uvr*A (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓	WP2 pKM101		UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay/Experiment 1 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

(b) Experiment 2 of the main mutation assay

Pre-incubation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 17 – 22 Mar 2010
Finalisation date: 07 Apr 2010

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 1.0 mL of molten overlay agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin or 2.5 mg/L tryptophan). The mixture was mixed in a test tube and poured onto selective agar plates. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for at least 48 hours at 37 °C in the dark. After incubation, the background bacterial lawn was examined and the number of bacterial colonies (his^+ or trp^+ revertants) was counted.

3. Pre-incubation test (PIT):
0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were mixed in a test tube and shaken at 37 °C for 60 minutes. After pre-incubation, 1.0 mL of molten overlay agar was added to each test tube. Each concentration and the controls were tested in triplicates. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ or trp^+ revertants) was counted.

4. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants (below the indication factor of 0.5 when compared to solvent controls)
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics
Results were judged without statistical analysis.

6. Acceptance criteria
The test was valid if

- There was a regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control were in the range of the laboratory's historical data.
- The positive control substances produced a significant increase in mutant colony frequencies.

7. Evaluation criteria
A test item was considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control was observed.

A dose dependent increase was considered biologically relevant if the threshold was exceeded at more than one concentration.
An increase exceeding the threshold at only one concentration was judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary toxicity test, there were no cytotoxic effects observed in any tester strain up to the highest tested concentration of 5000 µg/mL, neither in the presence nor the absence of metabolic activation. Since the second experiment was performed before the results of the preliminary experiment/experiment I were available, both experiments were performed with concentrations from 3 to 5000 µg/plate.

C. SOLUBILITY

Precipitation was observed only in the second experiment at 2500 and 5000 µg/mL in all strains in the presence of metabolic activation. There was no precipitation in the first experiment and no precipitation in the absence of metabolic activation.

D. MUTATION ASSAY

There was no substantial increase in the number of his^+ or trp^+ revertant colonies observed in any of the five tester strains following treatment with glyphosate technical spiked with glyphosine at any dose level, neither in the presence nor in the absence of metabolic activation.
The number of revertants induced by the vehicle control was within the range of the historical control data for each strain, thus demonstrating an acceptable experimental performance.
Appropriate positive control compounds showed a distinct increase in the number of revertant colonies, confirming the activity of the S9 mix and the validity of the test system.

Table B.6.4.1.4-1: *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine ([redacted] 2010), pre-experiment/first experiment

Preliminary experiment / Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	14	21	14	15	36	41	135	153	58	70
± SD	± 2	± 1	± 1	± 2	± 7	± 5	± 8	± 7	± 8	± 11
HCD[#] mean	15	18	12	16	31	39	142	154	53	62
± SD	± 3.41	± 4.69	± 3.23	± 3.92	± 5.67	± 6.91	± 21.88	± 25.80	± 8.10	± 8.80
[range]	7 - 36	8 - 55	5 - 27	7 - 31	14 - 59	16 - 84	85 - 226	94 - 239	34 - 80	32 - 87
Vehicle control										
Water mean	16	17	10	20	33	44	132	154	61	72
± SD	± 3	± 2	± 3	± 6	± 5	± 4	± 9	± 14	± 8	± 8
HCD[#] mean	16	19	12	15	30	38	132	144	52	61
± SD	± 3.37	± 4.37	± 2.84	± 3.59	± 5.26	± 6.58	± 23.21	± 25.42	± 8.11	± 8.66
[range]	8 - 38	10 - 41	6 - 27	7 - 33	15 - 52	16 - 59	94 - 218	94 - 241	33 - 76	34 - 82

Table B.6.4.1.4-1: *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine [redacted], 2010), pre-experiment/first experiment

Preliminary experiment / Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Test item [µg/plate]										
3 mean	14	21	10	20	34	47	131	164	57	70
± SD	± 4	± 5	± 3	± 4	± 4.6	± 3	± 6	± 10	± 10	± 11
10 mean	14	15	9	17	31	39	144	158	65	72
± SD	± 5	± 3	± 3	± 3	± 2.9	± 7	± 17	± 9	± 4	± 9
33 mean	15	16	8	21	33	45	149	165	59	70
± SD	± 2	± 5	± 1	± 2	± 4.7	± 5	± 6	± 8	± 7	± 3
100 mean	17	19	9	17	35	43	146	162	69	75
± SD	± 5	± 5	± 1	± 2	± 2.1	± 2	± 17	± 9	± 10	± 5
333 mean	11	17	11	20	31	43	131	166	59	76
± SD	± 3	± 2	± 4	± 1	± 2.6	± 7	± 20	± 4	± 9	± 5
1000 mean	13	14	13	14	32	41	140	169	68	65
± SD	± 4	± 1	± 1	± 5	± 8.5	± 7	± 8	± 5	± 1	± 4
2500 mean	15	14	8	11	34	50	129	155	47	65
± SD	± 1	± 1	± 1	± 2	± 6.1	± 1	± 14	± 5	± 7	± 8
5000 mean	12	15	9	11	26	40	98	92	35	43
± SD	± 0	± 4	± 4	± 3	± 2.0	± 6	± 16	± 18	± 7	± 15
Positive control										
[§] mean	1926	342	105	437	369	2072	1886	3249	1057	350
± SD	± 45	± 8	± 15	± 7	± 20	± 40	± 60	± 170	± 57	± 13
HCD[#] mean	1886	304	101	227	407	1586	1954	2032	808	332
± SD	± 242.09	± 154.47	± 28.30	± 67.24	± 98.13	± 454.52	± 426.94	± 569.62	± 434.27	± 154.50
[range]	663 - 2690	134 - 2404	58 - 440	68 - 498	216 - 897	198 - 3309	563 - 2844	594 - 3724	168 - 2528	175 - 1718

[§] Information on respective positive control is reported in Material and Method section I.A.2

[#] Historical control data generated from January - December 2009 in approx. 550 experiments (WP2uvrA approx. 300 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.

Table B.6.4.1.4-2: *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine ([redacted], 2010), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	16	21	11	16	30	46	155	169	54	67
± SD	± 4	± 3	± 3	± 1	± 9	± 4	± 9	± 12	± 7	± 8
HCD[#] mean	15	18	12	16	31	39	142	154	53	62

Table B.6.4.1.4-2: *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine ([redacted] 2010), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 3.41	± 4.69	± 3.23	± 3.92	± 5.67	± 6.91	± 21.88	± 25.80	± 8.10	± 8.80
[range]	7 - 36	8 - 55	5 - 27	7 - 31	14 - 59	16 - 84	85 - 226	94 - 239	34 - 80	32 - 87
Vehicle control										
Water mean	17	21	12	18	34	47	167	166	58	67
± SD	± 3	± 1	± 3	± 5	± 4	± 3	± 14	± 9	± 3	± 8
HCD[#] mean	16	19	12	15	30	38	132	144	52	61
± SD	± 3.37	± 4.37	± 2.84	± 3.59	± 5.26	± 6.58	± 23.21	± 25.42	± 8.11	± 8.66
[range]	8 - 38	10 - 41	6 - 27	7 - 33	15 - 52	16 - 59	94 - 218	94 - 241	33 - 76	34 - 82
Test item [µg/plate]										
3 mean	17	20	14	18	35	44	161	171	53	66
± SD	± 3	± 4	± 2	± 2	± 9	± 7	± 4	± 22	± 4	± 3
10 mean	18	18	14	19	34	45	155	157	55	73
± SD	± 3	± 4	± 4	± 4	± 4	± 8	± 17	± 12	± 1	± 12
33 mean	18	20	12	21	36	44	168	168	59	69
± SD	± 3	± 4	± 3	± 2	± 12	± 3	± 19	± 15	± 12	± 10
100 mean	18	23	11	20	35	45	150	175	55	66
± SD	± 3	± 3	± 3	± 1	± 4	± 4	± 2	± 9	± 2	± 12
333 mean	18	18	12	18[UM]	29	43	170	171	59	63[U]
± SD	± 4	± 3	± 3	± 5	± 1	± 10	± 11	± 17	± 8	± 13
1000 mean	15	23	14	18[UM]	32	49	157	165	56	64[U]
± SD	± 2	± 2	± 2	± 4	± 3	± 6	± 12	± 2	± 9	± 6
2500 mean	12	24[P]	15	12[UMP]	29	45[P]	138	149[P]	52	59[UP]
± SD	± 4	± 1	± 2	± 2	± 4	± 6	± 11	± 12	± 7	± 7
5000 mean	12	22[P]	10	11[UMP]	20	50[P]	129	100[P]	34	62[UP]
± SD	± 2	± 2	± 5	± 1	± 6	± 1	± 9	± 12	± 3	± 5
Positive control										
[§] mean	1688	431	123	547	453	2686	1985	3992	691	378
± SD	± 150	± 16	± 11	± 31	± 10	± 742	± 99	± 38	± 67	± 42
HCD[#] mean	1886	304	101	227	407	1586	1954	2032	808	332
± SD	± 242.09	± 154.47	± 28.30	± 67.24	± 98.13	± 454.52	± 426.94	± 569.62	± 434.27	± 154.50
[range]	663 - 2690	134 - 2404	58 - 440	68 - 498	216 - 897	198 - 3309	563 - 2844	594 - 3724	168 - 2528	175 - 1718
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated from January - December 2009 in approx. 550 experiments (WP2uvrA approx. 300 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[P] Precipitation observed										
[U] Air bubbles observed										

Table B.6.4.1.4-2: *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay with Solution of Glyphosate TC spiked with Glyphosine ([redacted] 2010), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[M] Manual count										

III. CONCLUSION

According to the results and under the experimental conditions of the present study, glyphosate technical spiked with glyphosine is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 *uvr*A) with and without metabolic activation.
The study was conducted under GLP conditions and according to OECD guideline 471 (1997) with no deviations. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate tech. spiked with glyphosine was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2uvrA) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.5. *In vitro studies – bacterial gene mutation, study 5*

Data point	CA 5.4.1/005
Report author	[redacted]
Report year	2010
Report title	Reverse Mutation Assay using bacteria (*Salmonella typhimurium*) with Glyphosate TC
Report No	101268
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) No. 440/2008 B.13/14 (2008), US EPA OPPTS 870.5100 (1998)
Deviations from current test guideline OECD 471 (1997)	2-Aminoanthracene was used as sole positive control in the presence of metabolic activation. Since the positive controls showed marked increases in the number of revertants, the deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

S. typhimurium strains TA 98, TA 100, TA 1535, TA 1537, and TA102 were exposed to glyphosate technical (batch: 200903051, purity: 98.2 %) in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction). Test item concentrations in the range of 31.6 to 5000 µg/plate were applied in two independent experiments using the plate-incorporation method (first experiment) and the pre-incubation method (second experiment). Sterility controls (untreated), vehicle (deionised water) and positive controls were included in each experiment. All conditions were tested in triplicates. After an exposure period of at least 48 h the bacterial background lawn was examined and the number of revertant colonies was counted.

There was no biologically relevant increase in the number of revertant colonies observed in any experiment at any tested concentration either in the presence or absence of metabolic activation. The spontaneous mutation rates of negative controls remained within the range of historical control data. Appropriate positive controls induced a

distinct response in the number of revertant colonies, thus demonstrating the functionality and validity of the test system. Based on the results and under the conditions of the present study, glyphosate is not mutagenic in the Ames test with and without metabolic activation.

V. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	/
Description:	Solid
Lot/Batch #:	200903051
Purity:	98.2 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until 26 Mar 2011.
Solvent (vehicle) used:	DMSO. Due to the low solubility of the test item stock solutions at 50 and 25 mg/mL were prepared and processed by ultrasound for 30 minutes at 37 °C.

2. Control materials:

Negative control:	Sterility controls were performed in accordance with the experimental design, but without addition of bacterial suspension.
Solvent (vehicle) control:	Deionised water and DMSO
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)	DMSO	2.5
TA 98	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)	DMSO	2.5
TA 1537	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	40.0
	+S9	2-Aminoanthracene (2-AA)	DMSO	2.5
TA 102	-S9	Methyl methane sulfonate (MMS)	Water	1.0
	+S9	2-Aminoanthracene (2-AA)	DMSO	10.0

3. Metabolic activation:

S9 mix was obtained from the livers of male Wistar rats that were induced (oral treatment) with 80 mg/kg bw phenobarbital and 100 mg/kg bw β-naphthoflavone for three consecutive days. Aliquots of the S9 mix were thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium ortho phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	5	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes)	✓
TA 1537	✓				
TA 102	✓				
TA 1538				Histidine auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

Plate-incorporation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 TA 102
Replicates:	Triplicates
Pre-incubation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 TA 102
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 25 Mar – 06 Apr 2010
Finalisation date: 08 Apr 2010

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten overlay agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin). All components were mixed and poured onto minimal agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C for at least 48 h in the dark, the background bacterial lawn was examined and the number of bacterial colonies (his^+ revertants) was counted.

3. Pre-incubation test (PIT):
0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were pre-incubated at 37 °C for 60 minutes. Afterwards, 2 mL of overlay agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin) were added and poured onto minimal agar plates. Each concentration and the controls were tested in triplicates. After solidification the plates were inverted and incubated for at least 48 h at 37°C in the dark. Subsequently, the background bacterial lawn was examined and the number of bacterial colonies (his^+ revertants) was counted.

4. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants down to a mutation factor of approximately ≤ 0.5 in relation to the solvent control
- clearing or diminution of the background lawn (= reduced his^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics
Statistical evaluation of the results was not regarded as necessary.

6. Acceptance criteria
The test was considered valid for each strain if

- The bacteria demonstrated their typical responses to ampicillin (TA 98, TA 100 and TA 102)
- The control plates with and without S9 mix (mean values of spontaneous reversion rates) were in the range of the laboratory's historical data
- Corresponding background growth on negative control, solvent control and test plates was observed

- The positive controls showed a distinct enhancement of revertant rates over the control plate.

7. Evaluation criteria

A test item was considered as a mutagen if the following criteria were met:

- There was a clear and dose-related increase in the number of revertants observed and/or
- There was a biologically relevant positive response for at least one of the dose groups observed in at least one tester strain with or without metabolic activation.

VI. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

Cytotoxicity was observed in the first experiment in strain TA100 at 5000 µg/plate and in strain TA1535 at ≥ 2500 µg/plate both in the presence and absence of metabolic activation. In the second experiment cytotoxicity was evident at 5000 µg/plate in tester strain TA100 without metabolic activation and in strain TA1535 with and without metabolic activation.

C. SOLUBILITY

There was no precipitation of the test substance in any tester strain observed up to the highest tested concentration, neither in the presence, not in the absence of metabolic activation.

D. MUTATION ASSAY

There was no biologically relevant increase in the number of revertant colonies observed in any experiment at any tested concentration either in the presence or absence of metabolic activation. The spontaneous mutation rates of negative controls remained within the range of historical control data and appropriate positive controls induced a distinct increase in the number of revertant colonies, thus demonstrating the functionality and validity of the test system.

Table B.6.4.1.5-1: Reverse Mutation Assay using bacteria (*Salmonella typhimurium*) with Glyphosate TC ([redacted], 2010), first experiment (plate-incorporation test)

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	21.0	22.0	104.0	109.0	9.0	9.0	12.0	12.0	290.0	186.0
± SD	± 2.3	± 5.9	± 12.7	± 9.0	± 2.1	± 1.0	± 2.5	± 4.2	± 8.5	± 5.1
DMSO mean	22.0	21.0	72.0	102.0	7.0	6.0	9.0	7.0	244.0	127.0
± SD	± 4.2	± 1.0	± 14.1	± 6.6	± 2.3	± 1.5	± 5.1	± 2.1	± 10.8	± 15.0
Negative control[N]										
HCD[#] mean	24.0	32.1	113.9	114.5	13.3	10.4	11.0	12.1	234.4	283.2
± SD	± 4.3	± 6.1	± 16.2	± 16.7	± 4.7	± 3.0	± 3.9	± 4.2	± 50.4	± 61.6
[range]	18 - 46	18 - 57	77 - 163	78 - 165	5 - 29	5 - 27	5 - 30	5 - 36	164 - 309	163 - 472
Test item [µg/plate]										
31.6 mean	23.0	30.0	87.0	123.0	7.0	6.0	11.0	11.0	235.0	141.0
± SD	± 1.2	± 8.1	± 11.1	± 10.3	± 3.2	± 2.1	± 3.8	± 2.1	± 10.1	± 9.5
100 mean	23.0	29.0	81.0	102.0	8.0	7.0	7.0	12.0	238.0	152.0
± SD	± 3.2	± 3.5	± 10.2	± 14.2	± 1.0	± 4.7	± 3.2	± 2.9	± 6.8	± 8.7
316 mean	19.0	30.0	81.0	100.0	6.0	5.0	10.0	9.0	218.0	135.0

Table B.6.4.1.5-1: Reverse Mutation Assay using bacteria (*Salmonella typhimurium*) with Glyphosate TC ([redacted], 2010), first experiment (plate-incorporation test)										
Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 3.5	± 2.1	± 10.6	± 7.5	± 1.2	± 3.1	± 1.0	± 2.1	± 7.1	± 7.0
1000 mean	18.0	28.0	75.0	119.0	5.0	6.0	12.0	10.0	229.0	167.0
± SD	± 2.3	± 3.1	± 15.7	± 17.2	± 0.6	± 2.5	± 2.9	± 2.0	± 13.0	± 10.8
2500 mean	22.0	31.0	72.0	98.0	1.0	3.0	7.0	9.0	183.0	130.0
± SD	± 0.6	± 3.6	± 12.3	± 8.7	± 1.0	± 2.1	± 3.0	± 2.6	± 17.8	± 16.4
5000 mean	15.0	21.0	12.0	51.0	0.0 [B]	1.0 [B]	5.0	8.0	141.0	74.0
± SD	± 4.0	± 4.5	± 3.5	± 13.6	± 0.0	± 1.2	± 3.8	± 1.0	± 7.8	± 12.7
Positive control										
[§] mean	361.0	1904.0	634.0	2373.0	543.0	132.0	110.0	347.0	1593.0	1160.0
± SD	± 12.1	± 384.8	± 256.6	± 99.6	± 326.6	± 27.9	± 14.6	± 12.7	± 172.1	± 119.3
HCD[#] mean	522.5	2378.0	1002.8	2083.8	1099.2	148.3	140.4	278.4	1601.4	1154.1
± SD	± 145.1	± 536.1	± 240.2	± 528.0	± 246.7	± 63.0	± 34.3	± 81.8	± 308	± 316.4
[range]	250 - 1508	260 - 3599	240 - 2307	500 - 3341	389 - 1827	31 - 687	43 - 453	58 - 502	550 - 2407	419 - 2102
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated in 2007 – 2009 (n = 566-921). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[N] Negative control historical control data combine solvent or vehicle as well as untreated controls										
[B] Background lawn reduced										

Table B.6.4.1.5-2: Reverse Mutation Assay using bacteria (*Salmonella typhimurium*) with Glyphosate TC ([redacted], 2010), second experiment (pre-incubation test)										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	22.0	29.0	118.0	125.0	8.0	11.0	11.0	9.0	351.0	271.0
± SD	± 5.9	± 4.7	± 9.3	± 9.5	± 3.0	± 2.3	± 3.0	± 2.9	± 7.1	± 17.0
DMSO mean	23.0	26.0	94.0	101.0	8.0	9.0	10.0	9.0	292.0	210.0
± SD	± 6.8	± 2.1	± 3.5	± 20.0	± 3.0	± 2.3	± 5.3	± 8.1	± 5.6	± 13.6
Negative control[N]										
HCD[#] mean	24.0	32.1	113.9	114.5	13.3	10.4	11.0	12.1	234.4	283.2
± SD	± 4.3	± 6.1	± 16.2	± 16.7	± 4.7	± 3.0	± 3.9	± 4.2	± 50.4	± 61.6
[range]	18 - 46	18 - 57	77 - 163	78 - 165	5 - 29	5 - 27	5 - 30	5 - 36	164 - 309	163 - 472
Test item [µg/plate]										
31.6 mean	20.0	28.0	110.0	112.0	14.0	7.0	8.0	9.0	233.0	195.0
± SD	± 2.9	± 6.7	± 7.6	± 19.4	± 3.8	± 3.6	± 2.1	± 0.6	± 15.8	± 12.2
100 mean	18.0	26.0	107.0	103.0	10.0	9.0	7.0	7.0	240.0	184.0
± SD	± 2.1	± 4.2	± 6.1	± 10.4	± 4.4	± 3.2	± 2.1	± 2.1	± 8.1	± 12.4

Table B.6.4.1.5-2: Reverse Mutation Assay using bacteria (*Salmonella typhimurium*) with Glyphosate TC ([redacted], 2010), second experiment (pre-incubation test)										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 102**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
316 mean	23.0	29.0	101.0	109.0	7.0	8.0	11.0	12.0	278.0	205.0
± SD	± 5.8	± 4.0	± 10.0	± 11.2	± 1.2	± 4.4	± 2.9	± 2.3	± 5.9	± 16.5
1000 mean	16.0	34.0	84.0	96.0	9.0	10.0	8.0	8.0	252.0	151.0
± SD	± 0.6	± 6.2	± 9.6	± 6.6	± 2.1	± 5.5	± 1.2	± 3.1	± 1.2	± 21.1
2500 mean	18.0	32.0	88.0	96.0	5.0	9.0	6.0	8.0	182.0	151.0
± SD	± 8.5	± 3.8	± 12.6	± 14.1	± 2.0	± 2.3	± 4.2	± 2.0	± 19.6	± 20.1
5000 mean	18.0	23.0	43.0	82.0	3.0	4.0	9.0	12.0	184.0	116.0
± SD	± 6.8	± 9.1	± 6.2	± 3.1	± 2.9	± 1.2	± 1.5	± 2.0	± 32.2	± 3.5
Positive control										
[§] mean	595.0	2172.0	722.0	2025.0	812.0	99.0	144.0	240.0	1747.0	1211.0
± SD	± 66.8	± 164.6	± 47.8	± 124.4	± 95.7	± 14.0	± 16.9	± 34.0	± 47.2	± 210.4
HCD[#] mean	522.5	2378.0	1002.8	2083.8	1099.2	148.3	140.4	278.4	1601.4	1154.1
± SD	± 145.1	± 536.1	± 240.2	± 528.0	± 246.7	± 63.0	± 34.3	± 81.8	± 308	± 316.4
[range]	250 - 1508	260 - 3599	240 - 2307	500 - 3341	389 - 1827	31 - 687	43 - 453	58 - 502	550 - 2407	419 - 2102
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated in 2007 – 2009 (n = 566-921). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[N] Negative control historical control data combine solvent or vehicle as well as untreated controls										

VII. CONCLUSION

Based on the results of the present study and under the experimental conditions of the test, the test item is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537 and TA102) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and under GLP conditions. There were only minor deviations, which were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate technical was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537, and TA 102) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.6. *In vitro studies – bacterial gene mutation, study 6*

Data point	CA 5.4.1/006
Report author	[redacted]
Report year	2009

Report title	Mutagenicity study of Glyphosate TC in the *Salmonella typhimurium* Reverse Mutation Assay (*in vitro*)
Report No	23916
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), Commission Regulation (EC) No. 440/2008 B.13/14 (2008), US EPA OPPTS 870.5100 (1998), ICH S2A (CPMP/ICH/141/95) and ICH S2B (CPMP/ICH/174/95)
Deviations from current test guideline OECD 471 (1997)	Historical control data for the positive controls were not included in the study report. Since the positive controls showed marked increases in the number of revertants, the deviation is not expected to significantly impact the study outcome. Furthermore, information in the study report on the HCD for the negative controls is limited.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** Some concerns were raised regarding the work conducted at this specific testing facility. After consultation with the responsible GLP monitoring authority, no GLP not in compliance (nic) reports on studies with glyphosate by this testing facility are available or known. As some deviations regarding the historical controls were identified (see above), the study is considered acceptable but with restrictions.

Glyphosate technical (batch: 20080801, purity 98.8 %) was investigated for its potential to induce gene mutations in bacteria in an Ames test. *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to the test item in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction) for 48 to 72 hours at 37 °C. Vehicle (aqua ad iniectabilia) and positive controls were included in each experiment.
Based on the results of a preliminary cytotoxicity test conducted in strain TA100 in the absence of metabolic activation, test item concentrations in the main mutagenicity test ranged from 31.6 to 3160 µg/plate. Two independent experiments were performed, using the standard plate test (plate-incorporation method) and the pre-incubation test. After 48 to 72 hours of incubation, the number of revertant colonies was counted and the bacterial background lawn was examined.
Precipitation of the test item was not reported. Cytotoxicity was observed for all tester strains at 3160 µg/plate in the presence and absence of metabolic activation.
There was no increase in the number of revertant his^+ colonies as compared to vehicle controls observed in any of the five tester strains following treatment with glyphosate technical tested up to concentrations of 3160 µg/plate, neither in the presence, nor in the absence of metabolic activation.
The number of revertants induced by the vehicle control was within the range of the laboratory's historical control data. The positive controls markedly induced the number of revertant colonies in each strain, demonstrating the functionality of the S9 mix and the sensitivity of the test system.
Based on the experimental results, glyphosate technical does not cause gene mutations in bacteria in the Ames standard plate and pre-incubation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate TC

Identification:	37/064/08
Description:	Solid, white
Lot/Batch #:	20080801
Purity:	98.8 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until the mentioned expiry date 01 Aug 2010. The stability of the test item in vehicle was not specified in the study report.
Solvent (vehicle) used:	Aqua ad iniectabilia

2. Control materials:

Negative control:	Sterility controls were not included in the present study
Solvent (vehicle) control:	Aqua ad iniectabilia
Solvent (vehicle)/final concentration:	0.1 mL per plate
Positive controls:	Please refer to the table below.

Strain	**Metabolic activation**	**Mutagen**	**Solvent**	**Conc. [µg/plate]**
S. typhimurium strains				
TA 100	-S9	Sodium azide	Water	10.0
	+S9	Cyclophosphamide	Water	1500.0
TA 1535	-S9	Sodium azide	Water	10.0
	+S9	Cyclophosphamide	Water	1500.0
TA 98	-S9	2-Nitrofluorene	DMSO	10
	+S9	2-Aminoanthracene	DMSO	2.0
TA 1537	-S9	9-Aminoacridine	Ethanol	100.0
	+S9	2-Aminoanthracene	DMSO	2.0
TA 102	-S9	Methylmethane sulfonate	DMSO	1300.0
	+S9	2-Aminoanthracene	DMSO	2.0

3. Metabolic activation:

S9 mix was obtained from the livers of 20 – 30 rats treated with Aroclor 1254. The pooled fraction exhibited a protein content of 31.55 mg/mL and a cytochrome P450 level of 0.41 nmol/protein. Aliquots of the S9 mix were thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100.0	mM
KCl	33.0	mM
NADPH-generating system		
Glucose 6-phosphate	5.4	mM
NADP	4.1	mM
$MgCl_2$	8.0	mM
S9	5.0	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB genes)	✓
TA 1537	✓				
TA 102	✓				
TA 1538				Histidine auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay

Plate incorporation test - S9 mix:	
Concentrations:	0.316, 1.0, 3.16, 10.0, 31.6, 100, 316, 1000, 3160 and 5000 µg/plate
Tester strain:	TA100
Replicates:	Duplicates

(b) Main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000 and 3160 µg/plate
Tester strains:	TA 98, TA 100, TA 102, TA 1535 and TA 1537
Replicates:	Triplicates
Pre-incubation test ± S9 mix:	
Concentrations:	31.6, 100, 316, 1000 and 3160 µg/plate
Tester strains:	TA 98, TA 100, TA 102, TA 1535 and TA 1537
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 04 – 27 Feb 2009
Finalisation date: 30 Apr 2009

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 phosphate buffer (in tests without metabolic activation) were added to 2.0 mL of molten overlay agar (supplemented with 0.5 mM L-histidine + 0.5 mM biotin). The test components were mixed by vortexing at low speed, poured onto coded minimal glucose agar plates and the plates were quickly tilted and rotated for homogenous distribution. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for 48 to 72 hours at 37 °C in the dark. After incubation, the number of revertant colonies was counted and the presence of bacterial background lawn was confirmed.

3. Pre-incubation test (PIT):
0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix phosphate buffer (in tests without metabolic activation) were pre-incubated for 20 minutes at 37 °C using a shaker.
Afterwards, 2.0 mL of molten overlay agar was added to each test tube and the mixture was poured on coded minimal glucose agar plates. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for 48 to 72 hours at 37 °C in the dark. After incubation, the number of revertant colonies was counted and the presence of bacterial background lawn was examined.

4. Cytotoxicity
Toxicity was detected by

- a reduction in the number of spontaneous revertants (below 50 % when compared to solvent controls)
- a clearing or diminution of the background lawn (= reduced his^- background growth)
- the degree of survival of the treated cultures

and recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics

Statistical significance for an increased number of revertants compared to the solvent control was determined using the Mann-Whitney U-test. A concentration-related effect was identified using the Spearman's rank correlation coefficient.

6. Acceptance criteria

Acceptance criteria were not defined in the study report.

7. Evaluation criteria

A test item was considered to show a positive response if the following criteria were met:

- The number of revertant colonies was statistically significantly increased compared to the solvent control to at least 2-fold of the solvent control for TA 98, TA 100 and TA 102 and 3-fold of the solvent control for TA 1535 and TA 1537 in both experiments.
- There was a statistically significant, concentration-related effect.
- Positive results were reproducible and the histidine independence of the revertants was confirmed by streaking random samples on histidine-free agar plates

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary toxicity test, cytotoxicity was noted in tester strain TA 100 at concentrations of 3160 and 5000 µg/plate in the absence of metabolic activation. Hence, 3160 µg/plate was chosen as top concentration for the main mutagenicity study.

In the main mutation assay, cytotoxicity (scarce background lawn) was noted for all tester strains at 3160 µg/plate in both experiments (plate-incorporation and pre-incubation test), both in the presence and absence of metabolic activation.

C. SOLUBILITY

Precipitation of the tests item was not reported.

D. MUTATION ASSAY

There was no increase in the number of revertant colonies as compared to control counts observed in any of the five tester strains following treatment with glyphosate technical tested up to 3160 µg/plate, neither in the presence nor in the absence of metabolic activation.

The number of revertants induced by the vehicle control was within the range of the historical control data for each strain. A marked increase in the number of revertant colonies was observed for the positive controls, demonstrating the functionality of the S9 mix and the sensitivity of the test system.

Table B.6.4.1.6-1: Mutagenicity study of Glyphosate TC in the Salmonella typhimurium Reverse Mutation Assay (in vitro) ([redacted], 2009), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Aqua ad iniectabilia mean	42.7	42.0	138.7	158.3	269.7	274.0	30.7	30.0	6.7	6.3
± SD	± 11.4	± 2.0	± 11.7	± 16.0	± 4.7	± 5.6	± 4.9	± 3.0	± 0.6	± 0.6
HCD[#] range	20 - 60		100 - 200		240 - 320		10 - 35		3 - 20	
Test item [µg/plate]										
31.6 mean	39.7	41.7	126.0	150.7	268.3	279.3	25.7	28.7	6.7	5.0

Table B.6.4.1.6-1: Mutagenicity study of Glyphosate TC in the Salmonella typhimurium Reverse Mutation Assay (in vitro) ([redacted], 2009), first experiment										
Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 7.6	± 8.3	± 7.0	± 32.7	± 2.5	± 5.7	± 2.5	± 1.5	± 1.2	± 1.0
100 mean	33.3	32.0	143.0	134.3	274.0	269.7	25.3	29.0	6.7	7.3
± SD	± 0.6	± 1.0	± 12.5	± 7.2	± 4.0	± 4.5	± 4.5	± 2.6	± 1.2	± 0.6
316 mean	30.7	42.0	143.0	132.7	259.0	274.7	28.7	28.3	4.7	6.0
± SD	± 1.5	± 7.0	± 4.6	± 2.1	± 3.6	± 5.5	± 3.2	± 3.8	± 0.6	± 1.0
1000 mean	30.0	32.3	124.0	128.7	266.0	272.0	30.3	32.7	4.7	5.7
± SD	± 1.7	± 11.0	± 13.1	± 9.5	± 7.0	± 5.3	± 0.6	± 3.2	± 1.5	± 1.5
3160 mean	36.6[$]	26.3[$]	109.0[$]	107.3[$]	259.0[$]	256.0[$]	28.7[$]	28.7[$]	4.3[$]	4.0[$]
± SD	± 2.1	± 4.0	± 3.6	± 2.1	± 3.6	± 4.4	± 2.5	± 3.2	± 0.6	± 1.0
Positive control										
[§] mean	393.7	387.0	720.3	730.7	1045.0	1159.3	366.7	375.0	364.0	375.3
± SD	28.6	± 10.5	± 31.2	± 9.3	± 19.2	± 31.4	± 8.1	± 12.5	± 21.0	± 24.6
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated in the laboratory of the testing facility (no further details available in the study report).										
[$] Cytotoxicity indicated by scarce background lawn										

Table B.6.4.1.6-2: Mutagenicity study of Glyphosate TC in the Salmonella typhimurium Reverse Mutation Assay (in vitro) ([redacted] 2009), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Aqua ad iniectabilia mean	40.3	52.0	150.0	152.3	267.0	274.3	18.7	19.7	4.3	6.7
± SD	± 6.8	± 12.2	± 6.2	± 9.7	± 12.5	± 16.9	± 6.1	± 3.5	± 0.6	± 0.6
HCD[#] range	20 - 60		100 - 200		240 - 320		10 - 35		3 - 20	
Test item µg/plate]										
31.6 mean	43.3	47.7	150.0	166.0	277.7	281.3	22.0	20.0	5.7	6.7
± SD	± 6.7	± 3.1	± 6.6	± 13.7	± 7.0	± 8.5	± 3.6	± 2.0	± 2.5	± 2.3
100 mean	44.3	50.7	145.3	164.0	275.0	268.7	16.3	19.7	6.3	6.3
± SD	± 5.7	± 1.5	± 24.6	± 1.0	± 9.0	± 2.5	± 2.3	± 5.5	± 1.2	± 0.6
316 mean	39.0	51.0	161.3	139.7	278.3	271.0	17.0	21.0	6.3	6.7
± SD	± 7.0	± 11.5	± 10.1	± 4.0	± 5.0	± 6.1	± 3.6	± 6.1	± 1.5	± 1.2
1000 mean	43.0	41.3	146.0	157.0	260.3	264.7	24.0	21.3	6.0	7.0
± SD	± 6.1	± 0.6	± 10.6	± 20.1	± 3.2	± 8.6	± 5.6	± 8.1	± 1.0	± 1.0
3160 mean	36.3[$]	44.7[$]	140.0[$]	161.0[$]	264.3[$]	262.0[$]	19.0[$]	18.0[$]	5.0[$]	5.7[$]
± SD	± 0.6	± 0.6	± 8.7	± 5.3	± 5.7	± 3.6	± 5.6	± 2.6	± 1.0	± 0.6
Positive control										
[§] mean	468.7	482.3	691.7	700.7	1178.3	1160.0	366.7	384.0	463.7	439.7

Table B.6.4.1.6-2: Mutagenicity study of Glyphosate TC in the Salmonella typhimurium Reverse Mutation Assay (in vitro) ([redacted], 2009), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 12.6	± 5.0	± 26.6	± 11.7	± 15.3	± 29.5	± 24.9	± 12.1	± 18.0	± 15.7
[§] Information on respective positive control is reported in Material and Method section I.A.2										
[#] Historical control data generated in the laboratory of the testing facility (no further details available in the study report).										
[$] Cytotoxicity indicated by scarce background lawn										

III. CONCLUSION:

According to the results and under the conditions of the present study, glyphosate technical is not mutagenic in the Ames standard plate and pre-incubation test with and without metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537) with and without metabolic activation.

The study was conducted according to OECD guideline 471 (1997) and in compliance with GLP. There were only minor deviations when compared to OECD 471 (1997) that do not hinder data evaluation. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:

It is agreed with the applicant's conclusion. Glyphosate technical was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537, and TA 102) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.7. *In vitro studies – bacterial gene mutation, study 7*

Data point	CA 5.4.1/007
Report author	[redacted]
Report year	2009
Report title	Glyphosate technical - *Salmonella typhimurium* and *Escherichia coli* Reverse Mutation Assay
Report No	1264500
Document No	Not reported
Guidelines followed in study	OECD 471 (1997), US EPA OPPTS 870.5100 (1998), Commission Regulation (EC) 2008/440 B13/B14 (2008)
Deviations from current test guideline OECD 471 (1997)	2-Aminoanthracene was used as sole positive control in the presence of metabolic activation, but the functionality of the S9 batch was routinely checked with benzo(a)pyrene according to the study author. Furthermore, the positive controls showed marked increases in the number of revertants. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strains WP2 uvrA (pKM101) and WP2 pKM101 were exposed to glyphosate technical (batch: 569753, purity: 96.3%) in the presence and absence of

metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for 48 hours at 37 °C. Vehicle (deionized water), sterility (untreated) and positive controls were included in each experiment.

A pre-experiment was conducted to identify cytotoxic concentrations of the test item. In a standard plate test (plate incorporation method), test item concentrations in the range of 3 to 5000 µg/plate did not show any cytotoxic effects in the presence and absence of metabolic activation. The pre-experiment was designated experiment I of the main mutation assay and analysed for the number of revertant colonies. A second main mutation assay was performed using the preincubation method and concentrations in the range of 33 to 5000 µg/plate. Both experiments were performed with triplicates. Following 48 hours of incubation, the mean number of revertant colonies was counted for each plate.

Precipitation of the test substance was not reported and there were no indications of cytotoxicity in any of the six tester strains up to the highest tested concentration of 5000 µg/plate.

There was no substantial increase in the number of his^+ or trp^+ revertants observed in any experiment at any dose level, neither in the presence nor absence of metabolic activation. In addition, there was no dose-dependent increase in mutation rates in the range below the generally acknowledged border of biological relevance.

The laboratory's historical control range was exceeded for tester strain WP2 uvrA pKM101 in the untreated and solvent control plates with and without S9 mix in the preliminary toxicity experiment/experiment I, and for tester strain WP2 uvrA pKM101 in the untreated control with S9 mix and the solvent control with and without S9 mix in experiment II. In strain WP2 pKM101 with and without metabolic activation in experiment I, the number of spontaneous revertants was below the lower limit of the laboratory historical control range. As discussed in the study report, the observations were considered to be the result of biologically irrelevant fluctuations in the number of colonies and were judged to have no detrimental impact on the outcome of the study. Since the number of revertants is consistent in all concentrations for these strains without major variability, and no increase of the number of revertant colonies was observed at all, the overall conclusion of an absence of a mutagenic effect is considered to be valid also for these tester strains.

Appropriate positive controls induced a marked increase in the number of revertants, confirming the activity of the S9 mix and the validity of the test system.

Based on the results of the present study and under the experimental conditions chosen, glyphosate technical is considered not mutagenic in the Ames standard plate and pre-incubation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	S1008322
Description:	Not specified
Lot/Batch #:	569753
Purity:	96.3 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) and in vehicle were not specified in the study report.
Solvent (vehicle) used:	Deionised water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Deionised water
Solvent (vehicle) /final concentration:	0.1 mL per plate
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 98	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1537	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	50.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
E. coli strains				
WP2 uvrA (pKM101)	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0
WP2 pKM101	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0

* The functionality of the S9 mix batch used was checked with benzo(a)pyrene and showed the expected results.

3. Metabolic activation:

S9 mix was obtained from the livers of 8 – 12 weeks old male Wistar rats, weighing approx. 220 - 320 g. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and oral administration of □-naphthoflavone (80 mg/kg bw) on three consecutive days. The livers were prepared 24 h after the last treatment. Aliquots of the S9 mix were thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)	✓	ampicillin resistance (R factor plasmid)	✓
TA 1535	✓	WP2 pKM101	✓	UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay/Experiment 1 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100, WP2 uvrA (pKM101) and WP2 pKM101
Replicates:	Triplicates

(b) Experiment 2 of the main mutation assay

Pre-incubation test ± S9 mix:	
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate

Tester strains:	TA 1535, TA 1537, TA 98, TA 100, WP2 uvrA (pKM101) and WP2 pKM101
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 23 Sep – 13 Oct 2009
   Finalisation date: 18 Dec 2009

2. **Standard plate test (plate-incorporation test, SPT):**

An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten overlay agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin or 2.5 mg/L tryptophan). The mixture was mixed in a test tube and poured onto selective agar plates. Each concentration and the controls were tested in triplicates. After solidification, the plates were incubated upside down for at least 48 hours at 37 °C in the dark. After incubation, the background bacterial lawn was examined and the number of the bacterial colonies (his^+ or trp^+ revertants) was counted.

3. **Pre-incubation test (PIT):**

0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were mixed in a test tube and shaken at 37 °C for 60 minutes. After pre-incubation, 2 mL of molten overlay agar was added to each test tube. Each concentration and the controls were tested in triplicates. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ or trp^+ revertants) was counted.

4. **Cytotoxicity**

Toxicity was detected by a

- reduction in the number of spontaneous revertants
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. **Statistics**

Results were judged without statistical analysis.

6. **Acceptance criteria**

The test was valid if

- There was a regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control were in the range of the laboratory's historical data.
- The positive control substances produced a significant increase in mutant colony frequencies.

7. **Evaluation criteria**

A test item was considered as a mutagen if a biologically relevant increased in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control is observed.

A dose dependent increase was considered biologically relevant if the threshold is exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration was judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary toxicity experiment, there were no indications of cytotoxicity up to the highest tested concentration of 5000 µg/plate. The preliminary experiment was designated as experiment I. Since no cytotoxic effects were observed, 5000 µg/plate were chosen as top concentration for experiment II. The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments. No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation

C. SOLUBILITY

There was no precipitation of the test substance observed up to the highest concentration tested.

D. MUTATION ASSAY

There was no substantial increase in the number of his^+ or trp^+ revertants observed in any of the six tester strains at any dose level, neither in the presence nor absence of metabolic activation. In addition, there was no dose-dependent increase in mutation rates in the range below the generally acknowledged border of biological relevance.

For tester strain WP2 uvrA pKM101 the laboratory's historical control range was exceeded in the untreated and solvent control plates with and without S9 mix, as well as most test item concentrations, in the preliminary toxicity experiment/experiment I, and in the untreated control with and without S9 mix and the solvent control with and without S9 mix in experiment II. In strain WP2 pKM101 the lower limit of the laboratory historical control range was not quite reached in the untreated and solvent controls with and without metabolic activation in experiment I and II except for the untreated control with S9 mix. At multiple test item concentrations in experiment II, the number of revertants were below the lower limit of the historical control data. As discussed in the study report, these elevated colony counts were considered to be the result of biologically irrelevant fluctuations in the number of colonies and were judged to have no detrimental impact on the outcome of the study.

Appropriate positive controls induced a marked increase in the number of revertants, confirming the activity of the S9 mix and the validity of the test system.

Table B.6.4.1.7-1: *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay ([redacted], 2009), pre-experiment/first experiment

Preliminary experiment / Experiment 1: Standard plate test (SPT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 pKM101**		**WP2 uvrA (pKM101)**	
Metabol. activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated												
mean	14	17	14	15	33	39	138	142	185	212	476	560
± SD	± 1	± 4	± 1	± 5	± 9	± 6	± 5	± 24	± 6	± 22	± 23	± 10
HCD# mean	17	20	13	18	31	39	139	147	380	441	273	322
± SD	± 5.33	± 6.23	± 3.38	± 4.05	± 5.45	± 6.53	± 17.30	± 21.78	± 42.63	± 50.20	± 39.53	± 54.45
[range]	9 - 38	10 - 46	5 - 26	8 - 31	16 - 55	19 - 59	93 - 205	92 - 234	255 - 446	285 - 512	188 - 339	216 - 440
Vehicle control												
DMSO mean	16	18	15	15	35	38	135	148	182	193	480	531
± SD	± 5	± 3	± 2	± 2	± 5	± 2	± 13	± 18	± 12	± 21	± 34	± 37
HCD# mean	17	21	13	17	30	39	130	155	374	406	251	281

Table B.6.4.1.7-1: *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay ([redacted], 2009), pre-experiment/first experiment

Preliminary experiment / Experiment 1: Standard plate test (SPT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 pKM101**		**WP2 uvrA (pKM101)**	
Metabol. activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 5.17	± 5.82	± 3.12	± 3.90	± 5.59	± 6.34	± 18.79	± 22.54	± 47.29	± 44.26	± 37.68	± 44.48
[range]	9 - 39	8 - 41	6 - 25	9 - 35	13 - 59	20 - 60	89 - 224	92 - 218	240 - 454	268 - 506	157 - 312	174 - 358
Test item [µg/plate]												
3 mean	14	16	14	16	28	37	138	157	193	197	486	536
± SD	± 5	± 4	± 1	± 3	± 4	± 6	± 18	± 14	± 17	± 26	± 27	± 22
10 mean	18	17	15	16	33	37	143	145	202	210	477	493
± SD	± 5	± 3	± 2	± 4	± 11	± 6	± 10	± 3	± 3	± 22	± 34	± 29
33 mean	13	16	16	16	29	40	135	157	188	212	499	545
± SD	± 2	± 3	± 2	± 1	± 2	± 3	± 2	± 14	± 5	± 42	± 38	± 22
100 mean	14	16	14	16	29	37	120	149	189	192	494	489
± SD	± 0	± 2	± 2	± 4	± 5	± 7	± 10	± 1	± 23	± 12	± 27	± 31
333 mean	15	19	16	18	31	35	128	159	207	204	484	196
± SD	± 2	± 3	± 2	± 3	± 3	± 6	± 7	± 14	± 11	± 25	± 20	± 15
1000 mean	14	17	12	16	30	33	138	140	170	179	451	517
± SD	± 3	± 6	± 4	± 1	± 6	± 5	± 10	± 12	± 15	± 14	± 22	± 26
2500 mean	11	15	13	14	24	32	122	132	167	189	428	456
± SD	± 3	± 3	± 3	± 1	± 5	± 3	± 8	± 7	± 31	± 29	± 9	± 23
5000 mean	10	20	14	18	27	30	89	108	110	136	427	442
± SD	± 2	± 4	± 1	± 3	± 4	± 2	± 12	± 7	± 16	± 6	± 19	± 4
Positive control												
[§] mean	1508	302	68	378	302	1188	1574	2215	2705	2333	2997	1930
± SD	± 52	± 50	± 4	± 105	± 4	± 9	± 118	± 144	± 106	± 145	± 332	± 138
HCD[#] mean	2024	294	116	204	489	1455	2160	1839	3058	1920	3020	1972
± SD	± 315.78	± 140.02	± 30.52	± 69.54	± 169.76	± 463.01	± 342.67	± 621.27	± 168.37	± 468.32	± 830.78	± 652.16
[range]	1041 3138	102 - 945	68 - 407	72 - 454	211 - 1694	200 - 3553	588 - 3379	404 - 3868	1369 - 5367	1163 - 3597	1522 - 4451	1043 - 3848

[§] Information on respective positive control is reported in Material and Method section I.A.2

[#] Historical control data generated from January - October 2008, representing approx. 600 experiments (approx. 150 experiments for WP2 uvrA pKM101; approx. 80 experiments for WP2 pKM101). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.

Table B.6.4.1.7-2: *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay ([redacted], 2009), second experiment

Experiment 2: Pre-incubation test (PIT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 pKM101**		**WP2 uvrA (pKM101)**	
Metabol. activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated												
mean	16	18	12	17	30	34	137	142	226	290	433	527
± SD	± 4	± 5	± 3	± 2	± 4	± 4	± 32	± 13	± 10	± 16	± 33	± 20
HCD[#] mean	17	20	13	18	31	39	139	147	380	441	273	322
± SD	± 5.33	± 6.23	± 3.38	± 4.05	± 5.45	± 6.53	± 17.30	± 21.78	± 42.63	± 50.20	± 39.53	± 54.45
[range]	9 - 38	10 - 46	5 - 26	8 - 31	16 - 55	19 - 59	93 - 205	92 - 234	255 - 446	285 - 512	188 - 339	216 - 440
Vehicle control												
DMSO mean	16	17	12	18	27	37	140	152	222	266	474	533
± SD	± 4	± 5	± 3	± 1	± 2	± 3	± 20	± 16	± 8	± 13	± 7	± 33
HCD[#] mean	17	21	13	17	30	39	130	155	374	406	251	281
± SD	± 5.17	± 5.82	± 3.12	± 3.90	± 5.59	± 6.34	± 18.79	± 22.54	± 47.29	± 44.26	± 37.68	± 44.48
[range]	9 - 39	8 - 41	6 - 25	9 - 35	13 - 59	20 - 60	89 - 224	92 - 218	240 - 454	268 - 506	157 - 312	174 - 358
Test item [µg/plate]												
33 mean	16	17	15	19	26	35	145	144	256	274	456	555
± SD	± 4	± 3	± 2	± 2	± 3	± 1	± 9	± 12	± 28	± 31	± 31	± 14
100 mean	17	18	11	18	29	33	151	152	239	274	456	603
± SD	± 4	± 2	± 3	± 2	± 4	± 4	± 7	± 6	± 7	± 21	± 6	± 29
333 mean	16	19	12	18	28	36	143	147	241	249	456	577
± SD	± 5	± 4	± 2	± 2	± 4	± 2	± 15	± 12	± 7	± 33	± 26	± 32
1000 mean	16	12	13	16	29	35	130	139	235	270	437	546
± SD	± 4	± 3	± 1	± 5	± 1	± 3	± 7	± 10	± 8	± 12	± 23	± 37
2500 mean	12	13	11	17	25	36	116	142	231	255	419	483
± SD	± 2	± 1	± 3	± 3	± 4	± 1	± 18	± 11	± 19	± 16	± 38	± 27
5000 mean	9	10	10	15	21	22	102	97	140	228	358	466
± SD	± 1	± 2	± 2	± 3	± 2	± 1	± 15	± 20	± 10	± 5	± 8	± 43
Positive control												
[§] mean	1521	297	80	237	366	1651	1673	1840	1657	1259	1777	2095
± SD	± 275	± 16	± 3	± 11	± 31	± 162	± 255	± 169	± 34	± 7	± 67	± 20
HCD[#] mean	2024	294	116	204	489	1455	2160	1839	3058	1920	3020	1972

Table B.6.4.1.7-2: *Salmonella Typhimurium* and *Escherichia Coli* Reverse Mutation Assay ([redacted] 2009), second experiment												
Experiment 2: Pre-incubation test (PIT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 pKM101**		**WP2 uvrA (pKM101)**	
Metabol. activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 315.78	± 140.02	± 30.52	± 69.54	± 169.76	± 463.01	± 342.67	± 621.27	± 168.37	± 468.32	± 830.78	± 652.16
[range]	1041 - 3138	102 - 945	68 - 407	72 - 454	211 - 1694	200 - 3553	588 - 3379	404 - 3868	1369 - 5367	1163 - 3597	1522 - 4451	1043 - 3848
§ Information on respective positive control is reported in Material and Method section I.A.2												
# Historical control data generated from January - October 2008, representing approx. 600 experiments (approx. 150 experiments for WP2 uvrA pKM101; approx. 80 experiments for WP2 pKM101). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.												

III. CONCLUSION:

Based on the results of the present study and under the experimental conditions of the test, the test item is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA (pKM101) and WP2 pKM101) with and without metabolic activation.

The study was performed under GLP conditions and compliant with OECD guideline 471 (1997), without any deviations. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:

It is agreed with the applicant's conclusion. Glyphosate technical was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2uvrA (pKM101) and WP2 pKM101) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

As already stated in the study summary, the number of revertants of strain WP2uvrA (pKM101) were above the upper limit of the historical control data of the negative control for the tested concentration, but also for the untreated and solvent controls. Since the number of these cultures are all exceeding the historical control data, and the fact that the number of revertants in the positive control is considerably higher, these observations are not considered biologically relevant and are considered to be normal fluctuations within the test system.

Furthermore, the number of revertants of strain WP2 (pKM101) was below the lower limit of the of the historical control data of the negative control, but this is also attributed to biological fluctuations and the observations are not considered biologically relevant.

B.6.4.1.8. *In vitro studies – bacterial gene mutation, study 8*

Data point:	CA 5.4.1/008
Report author	[redacted]
Report year	2008
Report title	Evaluation of the mutagenic potential of the test substance Glyphosate Technical by reverse mutation assay in *Salmonella typhimurium* (Ames Test)
Report No	RF-3996.401.392.07
Document No	Not reported
Guidelines followed in study	OECD 471 (1997)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)

Short description of study design and observations:	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 97a, and TA102 were exposed to glyphosate (batch: 20070606, purity: 98.05%) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Based on the results of a preliminary cytotoxicity test in tester strain TA100, in which cytotoxicity was observed at 2500 µg/plate in the absence of metabolic activation, concentrations in the range of 1 - 1000 µg/plate were selected for the main mutation assay. Triplicates of all tester strains were exposed via the plate-incorporation method to the test item for 72 hours. Sterility controls (untreated), vehicle (sterile water) and positive controls were included. Following incubation, the number of revertant colonies was counted for each strain.
Short description of results:	There was no clear evidence for cytotoxicity in any tester strain up to the highest concentration (1000 µg/plate) in the presence or absence of S9 mix, based on the number of revertant colonies. In addition, the test substance did not promote an increase in the number of revertant colonies in any strain either in the presence or absence of S9 mix. A statistically significant increase in the number of revertant colonies was observed with TA98 at 1 and 10 µg/plate and with TA100 at 500 µg/plate, both with S9 mix. However, the values were not increased by a factor of at least 2 compared to the values of the solvent controls and there was no dose-response relationship evident, therefore the findings were considered to be without relevance. The spontaneous mutation rates of negative controls remained within the range of historical control data and appropriate positive controls induced a marked increase in the number of revertants, thus demonstrating the functionality and validity of the test system. Based on the results of the present study and under the conditions of the test, glyphosate is not mutagenic in the Ames pre-incubation test with and without metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** No evidence of mutagenicity was obtained in the plate-incorporation assay up to 1000 µg/plate (highest concentration tested). As much lower concentrations were tested than in most other studies, the study was not considered as key study (and not accepted in RAR 2015). Category 3b **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since the test item was not tested at sufficiently high concentrations. Additional deviations that were noted: no HCD included in the study report; no repeat experiment was performed.

B.6.4.1.9. *In vitro studies – bacterial gene mutation, study 9*

Data point	CA 5.4.1/009
Report author	[redacted]
Report year	2007
Report title	*Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical (NUP-05068)
Report No	1061401
Document No	Not reported
Guidelines followed in study	OECD 471 (1997) referenced as EEC Directive 92/69 Method B13/B14; Japanese MAFF (2005)
Deviations from current test guideline OECD 471 (1997)	No reporting of bacterial cell density. 2-Aminoanthracene was used as sole positive control in the presence of metabolic activation, but the functionality of the S9 batch was routinely checked with benzo(a)pyrene according to the study author. Furthermore, the positive controls showed marked increases in the number of revertants. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)

GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strain WP2 uvrA were exposed to glyphosate technical (NUP-05068, batch: 200609062, purity: 95.1%) in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for 48 h. Untreated, vehicle (deionised water) and positive controls were included in each experiment. A pre-experiment was conducted to identify cytotoxic concentrations of the test item. Test item concentrations in the range of 3 to 5000 µg/plate did not show any cytotoxic effects in the presence and absence of metabolic activation. The pre-experiment was therefore reported as experiment I. A second independent experiment was performed, using the preincubation method with concentrations in the range of 33 to 5000 µg/plate.

There was no substantial increase in the number of his^+ and trp^+ revertant colony numbers in any of the experiments in any of the five tester strains at any dose level, neither in the presence nor absence of metabolic activation. The spontaneous reversion rate with and without metabolic activation was demonstrated with vehicle controls. Appropriate positive controls validated the sensitivity of the test system and the functionality of the metabolic activity of the S9 mix. Based on the results of the present study, glyphosate is not mutagenic in the Ames test with and without metabolic activation under the experimental conditions of the test.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	NUP-05068
Description:	Crystalline powder
Lot/Batch #:	200609062
Purity:	95.1%
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until 14 Sep 2008. The stability of the test item in solvent (vehicle) was not indicated by the sponsor.
Solvent (vehicle) used:	Deionised water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Deionised water
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 98	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1537	-S9	4-Nitro-o-phenylene-diamine (4-NOPD)	DMSO	50.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
E. coli strain				
WP2 uvrA	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0

* The functionality of the S9 mix batch used was additionally checked with benzo(a)pyrene and showed the expected results

3. Metabolic activation:

S9 mix was obtained from livers of 8 – 12 weeks old male Wistar HanIbm rats, weighing approx. 220 - 320 g. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and oral administration of □-naphthoflavone (80 mg/kg bw) on three consecutive days. The livers were prepared 24 h after the last treatment. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay/Experiment I (standard plate assay):

Plate incorporation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

(b) Experiment II (pre-incubation assay)

Pre-incubation test ± S9 mix:	
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 15 – 25 Jan 2007
   Finalisation date: 16 Mar 2007

2. **Standard plate test (plate-incorporation test, SPT):**

An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin or 2.5 mg/L tryptophan). The mixture was thoroughly shaken and poured onto minimal agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C for at least 48 h in the dark, the background bacterial lawn was examined and the bacterial colonies (his^+ or trp^+ revertants) were counted.

3. **Pre-incubation test (PIT):**

0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were mixed in a test tube and shaken at 37 °C for 60 minutes. After pre-incubation 2 mL of molten overlay agar was added to each test tube. Each concentration and the controls were tested in triplicates. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

4. **Cytotoxicity**

Toxicity was detected by a

- reduction in the number of spontaneous revertants
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. **Statistics**

Results were judged without statistical analysis.

6. **Acceptance criteria**

The test was valid if

- There was a regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control were in the range of the laboratory's historical data.
- The positive control substances produced a significant increase in mutant colony frequencies.

7. **Evaluation criteria**

A test item was considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and WP2 uvrA) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control was observed.

A dose dependent increase was considered biologically relevant if the threshold was exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration was judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary toxicity test, the test substance did not show any cytotoxicity to any strain up to the highest dose of 5000 µg/plate with and without metabolic activation.
In the second experiment, a slight reduction in the number of revertant colonies, was observed in tester strain TA 1537 at 5000 µg/plate in the absence of metabolic activation, indicating a weak cytotoxic effect of the test substance at the tested concentration.

C. SOLUBILITY

There was no precipitation of the test substance observed up to the highest dose tested.

D. MUTATION ASSAY

There was no relevant increase in the number of his^+ or trp^+ revertants observed in any experiment at any tested concentration, neither in the presence nor in the absence of metabolic activation. In addition, there was no dose-dependent increase in mutation rates in the range below the generally acknowledged border of biological relevance.

The number of revertants of strain WP2 uvrA induced by the vehicle control was slightly above the range of historical control data and for some test concentrations with S9 mix and for one test concentration without S9 mix in experiment I. The finding was judged to be based on biologically irrelevant fluctuations and was considered to have no impact on the outcome of the study. The absence of impact was confirmed by the negative results obtained in the second experiment.

Appropriate positive control compounds induced a marked increase in the number of revertants, demonstrating the validity of the test system and the functionality of the S9 mix.

Table B.6.4.1.9-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	18	24	13	19	20	31	147	162	68	81
± SD	± 8	± 4	± 4	± 6	± 8	± 3	± 18	± 7	± 9	± 14
HCD# mean	20.4	24.2	11.6	17.1	30.2	39	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	23	22	10	20	21	30	134	163	73	90
± SD	± 4	± 5	± 2	± 7	± 2	± 7	± 8	± 8	± 7	± 11
HCD# mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
3 mean	25.3	27	11	21	24	35	127	157	65	90
± SD	± 3	± 3	± 1	± 3	± 4	± 2	± 9	± 6	± 9	± 18
10 mean	16	22	13	20	24	44	132	153	79	97
± SD	± 5	± 6	± 5	± 5	± 5	± 4	± 14	± 9	± 10	± 5
33 mean	16	19	11	20	27	30	127	157	86	93
± SD	± 6	± 5	± 4	± 3	± 2	± 2	± 7	± 3	± 5	±17
100 mean	15	22	11	20	26	33	126	142	70	83

Table B.6.4.1.9-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted]i, 2007), first experiment										
Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 2	± 1	± 4	± 4	± 8	± 9	± 14	± 15	± 9	± 9
333 mean	23	22	9	15	25	38	145	147	63	88
± SD	± 3	± 11[#]	± 2	± 6	± 5	± 5	± 9	± 10	± 3	± 19
1000 mean	17	27	12	20	24	31	140	151	69	84
± SD	± 4	± 2	± 3	± 4	± 2	± 7	± 8	± 4	± 10	± 9
2500 mean	19	23	11	15	20	31	110	149	69	81
± SD	± 4	± 3	± 5	± 8	± 9	± 2	± 13	± 24	± 5	± 10
5000 mean	18	24	14	15	22	22	106	129	57	69
± SD	± 1	± 2	± 9	± 2	± 6	± 8	± 11	± 1	± 2	± 2
Positive control										
[§] mean	1885	398	100	301	378	1172	2060	2778	1558	269
± SD	± 55	± 21	± 4	± 7	± 10	± 116	± 80	± 91	± 86	± 15
HCD[#] mean	1422	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930
[§] Information on respective positive control is reported in Material and Method section I.A.2 [#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[#:] Contamination, analysis not possible in plate no. 2, mean of two plates										

Table B.6.4.1.9-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	17	20	13	17	29	30	143	185	54	68
± SD	± 4	± 1	± 8	± 6	± 9	± 7	± 1	± 7	± 11	± 5
HCD[#] mean	20.4	24.2	11.6	17.1	30.2	39	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	21	19	11	11	24	28	123	186	52	73
± SD	± 4	± 4	± 4	± 2	± 4	± 9	± 3	± 9	± 5	± 7
HCD[#] mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1

Table B.6.4.1.9-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
33 mean	16	15	14	18	26	31	133	180	61	81
± SD	± 2	± 5	± 2	± 3	± 4	± 6	± 3	± 9	± 4	± 10
100 mean	19	23	10	11	28	29	140	169	53	66
± SD	± 6	± 4	± 3	± 2	± 11	± 2	± 4	± 37	± 1	± 3
333 mean	19	22	10	15	26	30	140	191	44	69
± SD	± 5	± 6	± 4	± 5	± 2	± 2	± 11	± 29	± 4	± 5
1000 mean	19	23	9	15	28	29	143	192	50	67
± SD	± 6	± 6	± 3	± 6	± 8	± 6	± 13	± 17	± 7	± 9
2500 mean	17	22	10	11	26	23	112	163	48	53
± SD	± 2	± 6	± 5	± 3	± 2	± 8	± 4	± 5	± 4	± 3
5000 mean	11	23	4	11	20	24	95	126	25	55
± SD	± 3	± 9	± 3	± 4	± 9	± 2	± 10	± 4	± 12	± 12
Positive control										
[§] mean	1934	365	112	179	530	764	1861	2004	568	261
± SD	± 82	± 17	± 7	± 18	± 11	± 77	± 100	± 334	± 27	± 20
HCD[#] mean	1422.0	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930

[§] Information on respective positive control is reported in Material and Method section I.A.2
[#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.

III. CONCLUSION

In conclusion, under the experimental conditions reported, the test item (NUP-05068) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. There is no indication for mutagenicity in bacteria (Ames test) with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and under GLP conditions. There were only minor deviations when compared to the guideline, which were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate technical (NUP-05068) was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.10. *In vitro studies – bacterial gene mutation, study 10*

Data point	CA 5.4.1/010
Report author	[redacted]
Report year	2007
Report title	*Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical (NUP-05070)
Report No	1061402
Document No	Not reported
Guidelines followed in study	OECD 471 (1997) referenced as EEC Directive 92/69 Method B13/B14; Japanese MAFF (2005)
Deviations from current test guideline OECD 471 (1997)	No reporting of bacterial cell density. 2-Aminoanthracene was used as sole positive control in the presence of metabolic activation, but the functionality of the S9 batch was routinely checked with benzo(a)pyrene according to the study author. Furthermore, the positive controls showed marked increases in the number of revertants. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strain WP2 uvrA were exposed to glyphosate technical (NUP-05070, batch: 20060901, purity: 97.7 %) in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for 48 h. Untreated, vehicle (deionised water) and positive controls were included in each experiment. A pre-experiment was conducted to identify cytotoxic concentrations of the test item. In a standard plate test (plate incorporation method), cytotoxicity was observed as a reduced bacterial background lawn for strain TA 1537 at 333 – 5000 µg/plate in the presence of S9 mix, for strain TA 100 at ≥ 2500 µg/plate in the presence of S9 mix and for strain WP2 uvrA at 5000 µg/plate in the presence and absence of S9 mix.

The pre-experiment was designated experiment 1 of the main mutation assay. A second experiment was performed in the main mutation assay using the preincubation method and concentrations in the range of 33 to 5000 µg/plate. Both experiments were performed with triplicates. There was no reduced bacterial growth in the second experiment. Toxicity, evident as a reduced number of revertant colonies, was observed for strain WP2 uvrA at 5000 µg/plate without S9 mix in the first experiment and for strains TA 98 and WP2 uvrA at 5000 µg/plate in the presence and absence of S9 mix.

There was no substantial increase in the number of his^+ and trp^+ revertant colony numbers in any of the five tester strains at any dose level, neither in the presence nor absence of metabolic activation.

The spontaneous reversion rate with and without metabolic activation of the solvent (vehicle) control was within the range of historical control data. The sensitivity of the test system and the functionality of the S9 mix was demonstrated with appropriate positive controls. Based on the results of the present study and under the experimental conditions of the test, glyphosate is not mutagenic in the Ames test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification: NUP-05070

Description: Crystalline powder

Lot/Batch #: 20060901

Purity: 97.7 %

Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until 01 Sep 2008. The stability of the test item in solvent (vehicle) was guaranteed for 30 days at room temperature.
Solvent (vehicle) used:	Deionised water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Deionised water
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 98	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1537	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	50.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
E. coli strain				
WP2 uvrA	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0
* The functionality of the S9 mix batch used was additionally checked with benzo(a)pyrene and showed the expected results				

3. Metabolic activation:

S9 mix was obtained from the livers of 8 – 12 weeks old male Wistar HanIbm rats, weighing approx. 220 - 320 g. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and oral administration of □-naphthoflavone (80 mg/kg bw) on three consecutive days. The livers were prepared 24 h after the last treatment. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E. coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay/Experiment 1 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

(b) Experiment 2 of the main mutation assay

Pre-incubation test ± S9 mix:	
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 16 – 25 Jan 2007
   Finalisation date: 16 Mar 2007

2. **Standard plate test (plate-incorporation test, SPT):**

An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin or 2.5 mg/L tryptophan). The mixture was thoroughly shaken and poured onto minimal agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C for at least 48 h in the dark, the background bacterial lawn was examined and the bacterial colonies (his^+ or trp^+ revertants) were counted.

3. **Pre-incubation test (PIT):**

0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were mixed in a test tube and shaken at 37 °C for 60 minutes. After pre-incubation 2 mL of molten overlay agar was added to each test tube. Each concentration and the controls were tested in triplicates. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

4. **Cytotoxicity**

Toxicity was detected by a

- reduction in the number of spontaneous revertants
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. **Statistics**

Results were judged without statistical analysis.

6. **Acceptance criteria**

The test was valid if

- There was a regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control were in the range of the laboratory's historical data.
- The positive control substances produced a significant increase in mutant colony frequencies.

7. **Evaluation criteria**

A test item was considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and WP2 uvrA) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control was observed.

A dose dependent increase was considered biologically relevant if the threshold is exceeded at more than one concentration.
An increase exceeding the threshold at only one concentration was judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY
Cytotoxicity, indicated by a reduced background lawn was evident in the pre-test/first experiment in strain TA 1537 at concentrations in the range of 333 – 5000 µg/plate in the absence of S9 mix, in strain TA 100 at 2500 – 5000 µg/plate in the absence of S9 mix and in strain WP2 uvrA at 5000 µg/plate both in the presence and absence of S9 mix. There was no reduced bacterial background growth in the second experiment.

Toxic effects evident as a reduction in the number of revertant colonies was observed for strain WP2 uvrA at 5000 µg/plate without S9 mix in the first experiment and for strain TA 98 at 5000 µg/plate with S9 mix in the second experiment.

C. SOLUBILITY
There was no precipitation of the test substance observed up to the highest dose tested.

D. MUTATION ASSAY
No relevant increase in the number of his^+ or trp^+ revertants was observed in any experiment at any tested concentration either in the presence or absence of metabolic activation. In addition, there was no dose-dependent increase in mutation rates in the range below the generally acknowledged border of biological relevance.

The number of revertants detected in the vehicle control was within expected range for each strain, thus demonstrating an acceptable experimental performance. The untreated control in experiment 2 was slightly below the lower limit of the historical control data for strain TA 1535 with and without metabolic activation, however this is judged to be based on biologically irrelevant fluctuations in the number of colonies.

Appropriate positive control compounds induced a marked increase in the number of revertants, demonstrating the validity of the test system and the functionality of the S9 mix.

Table B.6.4.1.10-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted]i, 2007), pre-experiment/first experiment

Pre-experiment/Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	20	20	7	9	29	30	131	138	57	68
± SD	± 0	± 3	± 2	± 0	± 3	± 5	± 5	± 5	± 5	± 9
HCD# mean	20.4	24.2	11.6	17.1	30.2	39	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	18	24	14	13	27	33	131	140	61	78
± SD	± 1	± 3	± 2	± 9	± 4	± 7	± 9	± 11	± 12	± 10
HCD# mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9

Table B.6.4.1.10-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), pre-experiment/first experiment										
Pre-experiment/Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
3 mean	16	20	14	8	27	36	128	138	45	66
± SD	± 1	± 5	± 4	± 2	± 4	± 0	± 19	± 11	± 4	± 5
10 mean	21	21	12	9	31	37	138	149	51	68
± SD	± 2	± 1	± 2	± 2	± 2	± 3	± 11	± 7	± 4	± 9
33 mean	21	24	13	22	29	36	138	133	64	69
± SD	± 2	± 4	± 1	± 5	± 3	± 7	± 5	± 6	± 20	± 5
100 mean	17	23	15	21	31	41	127	147	56	65
± SD	± 6	± 8	± 6	± 1	± 4	± 7	± 16	± 6	± 7	± 6
333 mean	14	20	9 [R]	13	28	37	117	141	58	67
± SD	± 5	± 7	± 1	± 3	± 9	± 6	± 4	± 10	± 3	± 5
1000 mean	13	17	9 [R]	18	30	37	130	150	45	63
± SD	± 3	± 5	± 6	± 5	± 6	± 5	± 17	± 10	± 7	± 6
2500 mean	18	21	9 [R]	11	25	31	119 [R]	150	43	65
± SD	± 4	± 6	± 3	± 4	± 2	± 3	± 10	± 5	± 3	± 3
5000 mean	20	16	9 [R]	16	23	31	98 [R]	123	27 [R]	50 [R]
± SD	± 2	± 7	± 2	± 3	± 1	± 6	± 5	± 39	± 9	± 5
Positive control										
[§] mean	1935	309	82	81	503	1094	2447	1595	1324	241
± SD	± 62	± 19	± 5	± 6	± 3	± 33	± 87	± 107	± 69	± 13
HCD[#] mean	1422.0	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930
[§] Information on respective positive control is reported in Material and Method section I.A.2 [#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[R] Reduced background growth										

Table B.6.4.1.10-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										

Table B.6.4.1.10-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
mean	10 [BM]	9 [BM]	14	24	21 [BM]	43 [BM]	145	158	62	66
± SD	± 3	± 3	± 6	± 5	± 2	± 3	± 15	± 20	± 14	± 11
HCD[#] mean	20.4	24.2	11.6	17.1	30.2	39.0	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	9 [BM]	8 [BM]	16	24	21 [BM]	45 [BM]	130	211	53	69
± SD	± 1	± 1	± 5	± 8	± 4	± 5	± 3	± 29	± 2	± 6
HCD[#] mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
33 mean	8 [BM]	6 [BM]	13	24	16 [BM]	37 [BM]	131	180	54	80
± SD	± 2	± 1	± 1	± 12	± 2	± 8	± 5	± 15	± 6	± 9
100 mean	9 [BM]	11 [BM]	17	17	20 [BM]	38 [BM]	146	197	64	77
± SD	± 1	± 4	± 7	± 3	± 4	± 8	± 3	± 17	± 2	± 9
333 mean	10 [BM]	14 [BM]	18	18	18 [BM]	38 [BM]	152	183	65	67
± SD	± 3	± 2	± 3	± 3	± 2	± 1	± 5	± 20	± 7	± 7
1000 mean	7 [BM]	10 [BM]	16	19	18 [BM]	33 [BM]	150	156	49	64
± SD	± 1	± 1	± 4	± 5	± 2	± 3	± 2	± 20	± 12	± 9
2500 mean	8 [BM]	12 [BM]	14	22	14 [BM]	23 [BM]	137	163	34	68
± SD	± 1	± 3	± 3	± 2	± 2	± 6	± 27	± 11	± 6	± 29
5000 mean	9 [BM]	10 [BM]	10	18	12 [BM]	18 [BM]	127	130	30	49
± SD	± 3	± 2	± 6	± 2	± 2	± 4	± 12	± 6	± 5	± 7
Positive control										
[§] mean	2225	301	131	186	1012	1014	2234	1472	371	230
± SD	± 22	± 45	± 23	± 13	± 105	± 104	± 37	± 220	± 75	± 16
HCD[#] mean	1422.0	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930

[§] Information on respective positive control is reported in Material and Method section I.A.2

[#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.

[B] Extensive bacterial growth

[M] Manual count

III. CONCLUSION:

According to the results of the present study and under the conditions of the test, the test item (NUP-05070) is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and under GLP conditions. There were only minor deviations when compared to the guideline, which were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate technical (NUP-05070) was negative for gene mutation in bacteria (S. typhimurium TA 100, TA 98, TA 1535 and TA 1537 and E. coli WP2 uvrA in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.11. *In vitro studies – bacterial gene mutation, study 11*

Data point	CA 5.4.1/011
Report author	[redacted]
Report year	2007
Report title	*Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical (NUP-05067)
Report No	1061403
Document No	Not reported
Guidelines followed in study	OECD 471 (1997) referenced as EEC Directive 92/69 Method B13/B14; Japanese MAFF (2005)
Deviations from current test guideline OECD 471 (1997)	No reporting of bacterial cell density. 2-Aminoanthracene was used as sole positive control in the presence of metabolic activation, but the functionality of the S9 batch was routinely checked with benzo(a)pyrene according to the study author. Furthermore, the positive controls showed marked increases in the number of revertants. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

This study was performed to investigate the potential of glyphosate technical (NUP-05067, batch: 0609-1, purity: 95.0%) to induce gene mutations in *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strain WP2 uvrA. All strains were exposed, to the test item, vehicle (deionised water), untreated and appropriate positive controls in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction) for at least 48 h.

A pre-experiment was conducted to identify cytotoxic concentrations of the test item. In a standard plate test (plate incorporation method), the highest test item concentration (5000 µg/plate) induced cytotoxicity, evident as a reduced bacterial background growth, in strain WP2 uvrA in the absence of metabolic activation. The pre-experiment was designated experiment 1 of the main mutation assay and analysed for the number of revertant colonies. A second main mutation assay was performed using the pre-incubation method and concentrations in the range of 33 to 5000 µg/plate. Both experiments were performed with triplicates.

Minor cytotoxicity, evident as a reduction in the number of revertants, was observed at 5000 µg/plate for strain WP2 uvrA in the presence and absence of S9 mix in both experiments.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with the test item at any dose level, in any of the experiments, neither in the presence nor absence of

metabolic activation. The spontaneous reversion rate with and without metabolic activation of the solvent control was within the range of historical control data. A marked increase in the number of revertant colonies was obtained with appropriate positive controls, demonstrating the sensitivity of the test system and the functionality of the S9 mix. Based on the results of the present study and under the experimental conditions of the test, glyphosate is not mutagenic in the Ames test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	NUP-05067
Description:	Crystalline powder
Lot/Batch #:	0609-1
Purity:	95.0%
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until 15 Aug 2008. The stability of the test item in solvent (vehicle) was guaranteed for 30 days at room temperature.
Solvent (vehicle)used:	Deionised water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Deionised water
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	**Metabolic activation**	**Mutagen**	**Solvent**	**Conc. [µg/plate]**
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1535	-S9	Sodium azide (NaN_3)	Water	10.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 98	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	10
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
TA 1537	-S9	4-nitro-o-phenylene-diamine (4-NOPD)	DMSO	50.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	2.5
E. coli strain				
WP2 uvrA	-S9	Methylmethane sulfonate (MMS)	Water	3.0
	+S9	2-Aminoanthracene (2-AA)*	DMSO	10.0
* The functionality of the S9 mix batch used was checked with benzo(a)pyrene and showed the expected results.				

3. Metabolic activation:

S9 mix was obtained from the livers of 8 – 12 weeks old male Wistar HanIbm rats, weighing approx. 220 - 320 g. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and oral administration of β-naphthoflavone (80 mg/kg bw) on three consecutive days. The livers were prepared 24 h after the last treatment. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay/Experiment 1 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

(b) Experiment 2 of the main mutation assay

Pre-incubation test ± S9 mix:	
Concentrations:	33, 100, 333, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 16 – 25 Jan 2007
Finalisation date: 16 Mar 2007

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten agar (supplemented with 10.5 mg/L L-histidine + 12.2 mg/L biotin or 2.5 mg/L tryptophan). The mixture was thoroughly shaken and poured onto selective agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C for at least 48 h in the dark, the background bacterial lawn was examined and the bacterial colonies (his^+ or trp^+ revertants) were counted.

3. Pre-incubation test (PIT):
0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of S9 mix substitution buffer (in tests without metabolic activation) were mixed in a test tube and shaken at 37 °C for 60 minutes. After pre-incubation 2 mL of molten overlay agar was added to each test tube. Each concentration and the controls were tested in triplicates. The mixture was poured on selective agar plates. After solidification the plates were incubated upside down for at least 48 h at 37°C in the dark. Subsequently, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

4. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants

- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

5. Statistics

Results were judged without statistical analysis.

6. Acceptance criteria

The test was valid if

- There was a regular background growth in the negative and solvent control.
- The spontaneous reversion rates in the negative and solvent control were in the range of the laboratory's historical data.
- The positive control substances produced a significant increase in mutant colony frequencies.

7. Evaluation criteria

A test item was considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and WP2 uvrA) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control was observed.

A dose dependent increase was considered biologically relevant if the threshold was exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration was judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

Cytotoxicity indicated by a reduced background lawn was observed in the pre-test/first experiment in *E. coli* strain WP2 uvrA at 5000 µg/plate in the absence of metabolic activation. There was no reduced bacterial growth noted in the second experiment. Toxic effects evident as a reduction in the number of revertant colonies was observed at minor degree in both experiments at 5000 µg/plate in strain WP2 uvrA in the absence of metabolic activation.

C. SOLUBILITY

There was no precipitation of the test substance observed up to the highest dose tested.

D. MUTATION ASSAY

There was no substantial increase in the number of his^+ or trp^+ revertants observed in any experiment at any tested concentration either in the presence or absence of metabolic activation. In addition, there was no dose-dependent increase in mutation rates in the range below the generally acknowledged border of biological relevance.

The spontaneous mutation rates of negative controls remained within the range of historical control data and appropriate positive controls induced a marked increase in the number of revertants, thus demonstrating the functionality and validity of the test system.

Table B.6.4.1.11-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), pre-experiment/first experiment										
Pre-experiment/Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	19	21	11	20	33	41	139	135	53	72
± SD	± 8	± 5	± 4	± 4	± 6	± 7	± 16	± 2	± 10	± 10
HCD[#] mean	20.4	24.2	11.6	17.1	30.2	39.0	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	20	21	10	15	24	34	127	138	53	73
± SD	± 6	± 2	± 4	± 2	± 5	± 4	± 8	± 3	± 3	± 4
HCD[#] mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
3 mean	19	23	9	15	28	33	126	127	44	69
± SD	± 4	± 4	± 3	± 5	± 6	± 3	± 7	± 7	± 7	± 14
10 mean	20	22	10	16	28	41	136	139	54	77
± SD	± 6	± 3	± 2	± 0	± 4	± 9	± 11	± 4	± 6	± 2
33 mean	21	20	11	14	26	34	128	148	51	74
± SD	± 2	± 2	± 3	± 3	± 10	± 8	± 16	± 9	± 10	± 10
100 mean	20	16	12	12	25	32	131	148	51	71
± SD	± 7	± 7	± 4	± 4	± 2	± 7	± 8	± 1	± 6	± 14
333 mean	12	24	14	16	25	35	118	121	54	76
± SD	± 3	± 4	± 2	± 1	± 5	± 7	± 10	± 6	± 4	± 11
1000 mean	20	21	8	19	28	38	119	144	52	67
± SD	± 8	± 9	± 2	± 3	± 7	± 2	± 15	± 6	± 5	± 4
2500 mean	18	19	9	15	24	34	135	137	43	60
± SD	± 5	± 2	± 2	± 4	± 3	± 2	± 16	± 9	± 4	± 9
5000 mean	17	22	9	18	21	28	116	112	22 [R]	55
± SD	± 6	± 3	± 1	± 3	± 7	± 6	± 3	± 8	± 6	± 7
Positive control										
[§] mean	1991	345	102	208	436	1459	2196	2023	1304	322
± SD	± 41	± 11	± 9	± 9	± 43	± 133	± 97	± 40	± 54	± 16
HCD[#] mean	1422.0	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930
[§] Information on respective positive control is reported in Material and Method section I.A.2										

Table B.6.4.1.11-1: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), pre-experiment/first experiment										
Pre-experiment/Experiment 1: Standard plate test (SPT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.										
[R] Reduced background growth										

Table B.6.4.1.11-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment										
Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated										
mean	12	21	11	24	28	40	124	153	52	68
± SD	± 2	± 2	± 4	± 2	± 7	± 9	± 27	± 16	± 3	± 17
HCD[#] mean	20.4	24.2	11.6	17.1	30.2	39.0	138.2	150.1	55.9	65.6
± SD	± 4.4	± 5.5	± 4.0	± 5.4	± 6.6	± 7.5	± 21.6	± 24.2	± 8.6	± 10.4
[range]	11 - 31	10 - 38	4 - 28	7 - 34	16 - 60	18 - 64	86 - 216	96 - 214	36 - 76	33 - 91
Vehicle control										
Water mean	16	28	8	18	32	41	150	164	58	66
± SD	± 7	± 2	± 2	± 3	± 3	± 4	± 2	± 2	± 5	± 10
HCD[#] mean	20.8	24.7	11.2	16.2	28.1	37.9	130.7	147.0	55.8	63.9
± SD	± 4.7	± 5.9	± 3.7	± 5.0	± 6.1	± 7.4	± 20.8	± 25.5	± 7.2	± 9.1
[range]	9 - 35	7 - 43	5 - 28	6 - 36	15 - 49	20 - 57	87 - 197	84 - 255	31 - 74	34 - 84
Test item [µg/plate]										
33 mean	19	24	13	21	28	44	131	167	54	68
± SD	± 4	± 4	± 4	± 4	± 6	± 10	± 8	± 14	± 2	± 18
100 mean	16	22	14	22	26	36	144	161	54	62
± SD	± 6	± 8	± 1	± 8	± 3	± 14	± 7	± 11	± 11	± 4
333 mean	15	23	11	23	30	33	147	166	49	73
± SD	± 5	± 9	± 4	± 9	± 4	± 3	± 3	± 19	± 12	± 9
1000 mean	13	21	10	21	30	39	148	154	41	67
± SD	± 4	± 2	± 4	± 2	± 4	± 9	± 8	± 32	± 7	± 7
2500 mean	19	19	10	19	24	39	135	153	30	49
± SD	± 3	± 8	± 1	± 8	± 3	± 13	± 3	± 11	± 4	± 9
5000 mean	10	21	9	21	25	42	105	146	24	49
± SD	± 3	± 2	± 2	± 2	± 9	± 8	± 13	± 34	± 11	± 5
Positive control										
[§] mean	2045	335	105	229	356	1008	2246	2331	705	287

Table B.6.4.1.11-2: *Salmonella typhimurium* and *Escherichia coli* Reverse mutation assay with Glyphosate technical ([redacted], 2007), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 99	± 12	± 5	± 45	± 28	± 128	± 39	± 280	± 40	± 18
HCD[#] mean	1422.0	332.0	99.8	276.8	439.0	1839.4	2083.1	2372.9	991.0	319.4
± SD	± 464.7	± 95.3	± 32.5	± 132.6	± 155.2	± 898.6	± 281.3	± 958.4	± 522.9	± 84.8
[range]	781 - 4900	107 - 695	53 - 425	59 - 746	176 - 1818	407 - 4891	616 - 2872	417 - 5230	249 - 1810	211 - 930

[§] Information on respective positive control is reported in Material and Method section I.A.2
[#] Historical control data from May 2005 – June 2006 representing approx. 200 experiments (for WP2 uvrA approx. 100 experiments). It is unclear whether data are from standard plate tests, pre-incubation tests, or from a combined dataset.

III. CONCLUSION:

Based on the results of the present study and under the experimental conditions of the test, the test item is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and under GLP conditions. There were only minor deviations when compared to the guideline, which were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate technical (NUP-05067) was negative for gene mutation in bacteria (S. typhimurium TA 100, TA 98, TA 1535 and TA 1537 and E. coli WP2 uvrA in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.12. *In vitro studies – bacterial gene mutation, study 12*

Data point	CA 5.4.1/012
Report author	[redacted]
Report year	2007
Report title	Bacterial reverse mutation test (Ames Test) for Glifosato Téchnico
Report No	RL3393/2007-2.0AM-B
Document No	Not reported
Guidelines followed in study	OECD 471 (1997)
Deviations from current test guideline OECD 471 (1997)	2-Aminoanthracene was used as sole positive control in the presence of metabolic activation. Since a marked increase in the number of revertants was observed for the positive controls, this deviation is accepted. Only a single experiment was performed, without giving a justification for not conducting a confirmatory experiment. Historical control data were restricted to solvent controls, but not differentiated for exposures in the presence or absence of metabolic activation.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes

Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be not acceptable.

S. typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 102 were exposed to glyphosate technical (batch: 2007091801, purity: 98.01 %) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Based on the results of a preliminary cytotoxicity test, in which no toxicity was observed up to 5000 µg/plate, concentrations for the main mutation assay were selected. In the main experiment the tester strains were exposed via the plate-incorporation method to test item concentrations in the range of 648 to 5000 µg/plate for 72 hours. Vehicle (DMSO) and positive controls were included. Following incubation, the bacterial background lawn was examined and the number of revertant colonies was counted for each strain.

Precipitation of the test substance was not reported. Cytotoxicity as indicated by a statistically significant reduction in the number of revertant colonies was observed at 5000 µg/plate in strain TA 1537 in the absence of S9 mix.

There was no substantial increase in the number of his^+ and trp^+ revertants (exceeding a mutation rate by a factor of at least 2 when compared to solvent controls) observed in any experiment either in the presence or absence of metabolic activation and no dose-response relationship in the range below the generally acknowledged border of biological significance. Vehicle controls were within the range of historical control data and positive controls demonstrated the sensitivity and the functionality of the test substance.
Based on the results of the present study, glyphosate is not mutagenic in the Ames pre-incubation test with and without metabolic activation under the experimental conditions of the test.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	Glifosato Téchnico Helm
Description:	Solid
Lot/Batch #:	2007091801
Purity:	98.01 %
Stability of test compound:	The stability of the test item under storage conditions was guaranteed until 17 Sep 2009. In addition, it was confirmed by CIPAC MT 46 at 54 °C for 14 days
Solvent (vehicle) used:	Dimethylsulfoxide (DMSO)

2. Control materials:

Negative control:	Not examined
Solvent (vehicle) control:	Dimethylsulfoxide (DMSO)
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Conc. [µg/plate]
TA 100	-S9	Sodium azide	5.0
	+S9	2-Aminoanthracene	2.5
TA 1535	-S9	Sodium azide	5.0
	+S9	2-Aminoanthracene	2.5
TA 98	-S9	2-Nitrofluorene	0.5
	+S9	2-Aminoanthracene	2.5
TA 1537	-S9	ICR 191-Acridine	10.0
	+S9	2-Aminoanthracene	2.5
TA 102	-S9	Mitomycin C	0.5
	+S9	2-Aminoanthracene	2.5

3. Metabolic activation:

S9 mix was purchased from [redacted] The homogenate was produced from the livers of rats which were induced with Aroclor 1254. The concentration of protein in S9 fraction employed in the assay was 34.9 mg/mL.

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes)	✓
TA 1537	✓				
TA 102	✓				
TA 1538				Histidine auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay:

Plate incorporation test without S9 mix:	
Concentrations:	8, 40, 200, 1000 and 5000 µg/plate
Tester strains:	TA 100
Replicates:	Triplicates

(b) Mutation assay:

Plate incorporation test ± S9 mix:	
Concentrations:	648, 1080, 1800, 3000 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates:	Triplicates in a single experiment.

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 06 Nov – 03 Dec 2007
Finalisation date: 13 Dec 2007

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 3 mL of top agar. Each suspension was incubated on selective agar plates for 72 h. After incubation the background bacterial lawn was examined and the bacterial colonies (his^+ or trp^+ revertants) were counted.

3. Cytotoxicity
Cytotoxicity was investigated in a preliminary dose-range finding study, which was performed in tester strain TA100 in the absence of metabolic activation. Toxicity was detected by a

- decrease in the number of revertants
- clearing or diminution of the background lawn (= reduced his^- background growth)

and recorded for all test groups both with and without S9 mix.

4. Statistics
Analysis of variance (ANOVA) was performed on the data to identify statistical significance (p ANOVA < 0.05)

5. Acceptance criteria
The following acceptance criteria were defined:

- Presence of background lawn in the test plates.
- Spontaneous revertant colonies of the negative control were in the range reported in literature and established in the laboratory by historical control values.
- Positive controls showed mutagenicity in all tested strains.

6. Evaluation criteria

Results were judged positive when the following criteria were met:

- The mutation rates after 72 hours of incubation of strains exposed to the test chemical were higher than 2 for strains TA 98, TA 100 and TA 102 or higher than 3 for strains TA 1535 and TA1537.
- The positive result was statistically significant (pANOVA < 0.05) and a clear dose-related increase in the number of revertants was observed.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary cytotoxicity experiment, the test substance did not show any toxicity to any strain up to the highest dose tested, neither in the presence, nor in the absence of metabolic activation.

According to the result tables of the study report, in the main experiment, cytotoxicity as indicated by a statistically significant reduction in the mean number of revertant colonies, was observed at 5000 µg/plate in strain TA 1537 in the absence of S9 mix. There was no cytotoxicity observed in any other strain, neither in the presence, nor absence of S9 mix. These observations on cytotoxicity listed in the result tables of the study report are inconsistent with the observations described in the text of the study report, where a reduction in the mean number of revertant colonies was observed at 5000 µg/plate for strain TA 1537 in the presence of S9 mix and for strain TA 102 in the absence of S9 mix.

C. SOLUBILITY

Precipitation of the test substance was not reported.

D. MUTATION ASSAY

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment at any concentration level, neither in the presence nor absence of metabolic activation. There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological significance.

The number of revertants induced by the vehicle control was within expected range of historical control data and data published in literature for each strain, thus demonstrating an acceptable experimental performance.

Appropriate positive control compounds induced a marked increase in the number of revertants, demonstrating the functionality of the S9 mix and the validity of the test system.

Table B.6.4.1.12-1: Bacterial reverse mutation test (Ames Test) ([redacted], 2007)

Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
DMSO mean	34	32	204	203	312	307	33	30	12	9
± SD	± 3	± 3	± 4	± 2	± 4	± 6	± 3	± 2	± 1	± 2
HCD[#] [range]	17-75		60-220		240-320		5-50		3-25	
Test item [µg/plate]										
648 mean	29	30	209	204	311	312	32	28	10	14
± SD	± 2	± 2	± 8	± 4	± 6	± 4	± 2	± 4	± 2	± 2
1080 mean	32	34	203	204	309	307	33	31	10	14
± SD	± 4	± 4	± 5	± 4	± 6	± 5	± 2	± 4	± 3	± 5
1800 mean	33	37	203	209	308	305	29	28	9	8

Table B.6.4.1.12-1: Bacterial reverse mutation test (Ames Test) ([redacted], 2007)										
Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 102**		**TA 1535**		**TA1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	± 3	± 7	± 2	± 8	± 8	± 6	± 1	± 2	± 2	± 1
3000 mean	31	35	208	213	308	304	34	33	13	7
± SD	± 6	± 2	± 8	± 6	± 4	± 4	± 3	± 2	± 4	± 2
5000 mean	34	36	209	208	287	304	33	28	7	9
± SD	± 2	± 5	± 8	± 11	± 9	± 4	± 5	± 5	± 2	± 4
Positive control[$]										
[§] mean	1076	3290	2926	2828	3136	1890	2576	272	3501	258
± SD	± 62	± 337	± 99	± 79	± 317	± 218	± 119	± 28	± 120	± 8
[§] Information on respective positive control is reported in Material and Method section I.A.2 [#] Historical control data, time frame of generation not specified. HCD not differentiated for exposures in the presence or absence of S9 mix. No HCD available for the positive controls. [$] The positive control was tested in duplicates only.										

III. CONCLUSION:

According to the results of the present study and under the experimental conditions of the test, the test item is not mutagenic in the Ames plate-incorporation test with and without metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 1535, TA 1537 and TA 102) with and without metabolic activation.

The study was performed under GLP conditions and according to OECD guideline 471 (1997), including some deviations from the guideline. No justification was provided for conducting only a single experiment according to the plate incorporation method, however, given the clearly negative result, this is considered acceptable. Further deviations to OECD guideline 471 (1997) were considered to be of minor degree and to not compromise the validity of the study. The study was considered to provide supporting information.

Assessment and conclusion by RMS:

It is agreed with the applicant's conclusion. Glyphosate technical was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537, and TA 102) in the presence and absence of metabolic activation under the conditions of this test. In contrast to the applicant and the previous evaluation (RAR, 2015), the study is not considered to be acceptable because no confirmatory experiment was performed.

B.6.4.1.13. *In vitro studies – bacterial gene mutation, study 13*

Data point	CA 5.4.1/013
Report author	[redacted]
Report year	1996
Report title	Glyphosate Acid: An Evaluation of Mutagenic Potential Using *S. typhimurium* and *E. coli*
Report No	CTL/P/4874
Document No	YV3611
Guidelines followed in study	OECD 471 (1983); OECD 472 (1983); U.S. EPA FIFRA Guidelines, Subdivision F (1991); EEC Directive 92/69 Method B.13/B14 (1992)
Deviations from current test guideline OECD 471 (1997)	No historical control data provided. No information on cytotoxicity reported. Evaluation of cytotoxicity and precipitation were not reported, but concentrations were tested up to limit doses. 2-Aminoanthracene was used as sole indicator of the efficacy of the S9 mix in all strains. No confirmation of bacterial cell density.

Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the deviations noted above.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strains WP2P (WP2 pKM101) and WP2 uvrA were exposed to glyphosate acid (batch: P24, purity: 95.6%) in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction). The test was performed using the plate-incorporation method (first experiment) and the pre-incubation method (second experiment) and concentrations in the range of 100 – 5000 µg/plate. Solvent (DMSO) and positive controls were included in each experiment. After 72 hours of incubation at 37 °C, the bacterial background lawn was inspected and the number of revertant colonies was examined.

Evaluation of precipitation and cytotoxicity was not provided in the study report. Cytotoxicity was evaluated retrospectively by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding solvent control.

There was no statistically significant, reproducible increase in in the number of his^+ and trp^+ revertant colony numbers in any of the six tester strains at any dose level, neither in the presence nor absence of metabolic activation. Statistical significance in the mean number of revertant colonies was observed for tester strains TA 100 and WP2P (WP2 pKM101) at single concentrations in the presence or absence of S9 mix, however, the results were not reproducible in independent experiments and showed no dose-response relationship and were thus considered to be incidental. The spontaneous reversion rate with and without metabolic activation was demonstrated with vehicle controls. Appropriate positive controls validated the sensitivity of the test system and the functionality of the metabolic activity of the S9 mix.

Based on the results and under the experimental conditions of the present study, there is no indication for mutagenicity in the Ames standard plate and pre-incubation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate acid

Identification:	Y04707/034
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6%
Stability of test compound:	The stability of the test item under storage conditions (at ambient temperature in the dark) was guaranteed until the stated expiry date (no date provided). The stability of the test item in solvent (vehicle) was not indicated by the sponsor.
Solvent (vehicle) used:	Dimethylsulfoxide (DMSO)

2. Control materials:

Negative control:	Not specified
Solvent (vehicle) control:	DMSO
Solvent (vehicle)/final concentration:	10 µL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	Sodium azide (NaN_3)	DMSO	0.5, 1.0 and 2.0
	+S9	2-Aminoanthracene (2AA)	DMSO	0.2, 0.5 and 1.0
TA 1535	-S9	Sodium azide (NaN_3)	DMSO	0.5, 1.0 and 2.0
	+S9	2-Aminoanthracene (2AA)	DMSO	0.5, 1.0 and 2.0
TA 98	-S9	Daunorubicin Hydrochloride (DR)	DMSO	0.2, 0.5 and 1.0
	+S9	2-Aminoanthracene (2AA)	DMSO	0.2, 0.5 and 1.0
TA 1537	-S9	Acridine Mutagen ICR191 Dihydrochloride (ACM)	DMSO	0.5, 1.0 and 2.0
	+S9	2-Aminoanthracene (2AA)	DMSO	0.5, 1.0 and 2.0
E. coli strains				
WP2 uvrA	-S9	N-Ethyl-N-nitro-N-nitrosoguanidine (ENNG)	DMSO	0.2, 0.5 and 1.0
	+S9	2-Aminoanthracene (2AA)	DMSO	1, 2 and 5
WP2P (WP2 pKM101)	-S9	Mitomycin C (MMC)	DMSO	0.2, 0.5 and 1.0
	+S9	2-Aminoanthracene (2AA)	DMSO	5, 10 and 20

3. Metabolic activation:

S9 mix was obtained from the livers of male Sprague-Dawley rats, dosed once daily by oral gavage for three days with a combined phenobarbital (80 mg/kg bw) and β-naphthoflavone (100 mg/kg bw) corn oil solution. The livers were prepared one day after the third dose. A 25% (w/v) homogenate fraction was prepared using sucrose-Tris-EDTA buffer (250:50:1 mM), adding 3 mL S9 fraction to 7 mL buffer. Afterwards 20 mL co-factor solution were immediately added containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	150	mM
KCl	49.5	mM
NADPH-generating system		
Glucose 6-phosphate	7.55	mM
NADP, Na salt	6	mM
$MgCl_2$	12	mM
S9	25	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2P (WP2 pKM101)	✓	ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

Plate incorporation test first experiment ± S9 mix; second experiment -S9 mix:	
Concentrations:	100, 200, 500, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100, WP2P (WP2 pKM101) and WP2 uvrA
Replicates:	Triplicates
Pre-incubation test + S9 mix (second experiment only):	
Concentrations:	100, 200, 500, 1000, 2500 and 5000 µg/plate
Tester strains:	TA 1535, TA 1537, TA 98, TA 100, WP2P (WP2 pKM101) and WP2 uvrA

Replicates:	Triplicates

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 28 Nov – 11 Dec 1995
   Finalisation date: 16 Feb 1996

2. **Standard plate test (plate-incorporation test, SPT):**

An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial overnight culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 substitution buffer (in tests without metabolic activation) were added to 2 mL of molten agar (supplemented with 0.5 mg histidine/0.5 mM biotin stock solution (10 mL solution : 100 mL agar) or 10 mL 0.5 mM stock tryptophan per 100 mL agar). The mixture was thoroughly shaken and poured rapidly onto Vogel Bonner agar plates. Each concentration and the controls were tested in triplicates. After incubation at 37 °C for 72 h in the dark, the plates were checked for microbial contamination, the background bacterial lawn was examined and the number of the bacterial colonies (his^+ or trp^+ revertants) was counted.

3. **Pre-incubation test (PIT):**

The assay procedure was as for the plate-incorporation protocol as described above, except that a) where possible, each compound/solvent dose was added in 0.02 ml volumes, with the total volume made up to 0.1ml with phosphate buffered saline and b) Before adding the top agar, each compound/strain group of bijoux were placed on an orbital shaker (at approximately 140rpm) for 60 minutes (at 37°C).

4. **Cytotoxicity**

Evaluation of cytotoxicity was not further specified in the study report. Thus, retrospectively, cytotoxicity was evaluated by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding solvent control. Cytotoxicity was considered evident when the mean number of revertant colonies induced by the test item concentration was ≤ 0.5 fold of the mean number of spontaneous revertant colonies induced by the solvent control.

5. **Statistics**

An assessment of statistical significance was carried out using a one-tailed Student's t-test. Values of $p < 0.01$ are treated as significant with values of $0.01 \leq p < 0.05$ being indicative of a possible effect.

6. **Acceptance criteria**

The test was valid if

- The concurrent solvent control data were acceptable
- The positive control data showed unequivocal positive responses.

Failure of one or more tester strain/S9 combinations does not invalidate the data for the remainder of a concurrent experiment.

7. **Evaluation criteria**

A positive response in an individual experiment was achieved when one or both of the following criteria were met:

- A statistically significant dose-related increase in the mean number of revertant colonies was obtained.
- A two-fold or greater increase of statistical significance in the mean number of revertant colonies was observed at one or more concentrations.

For a positive response in an individual experiment to be considered indicative of an unequivocal positive, i.e. mutagenic, result for that strain/S9 combination, then the observed effects must be consistently reproducible.

A negative response in an individual experiment was achieved when

- There was no statistically significant dose-related increase in the mean number of revertant colonies per plate observed for the test substance.
- In the absence of any such dose response, no increase in colony numbers was observed at any test concentration, which exceeded 2x the concurrent solvent control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

Indications for cytotoxicity were observed in the first experiment in tester strains TA 1535, TA 98 and TA 100 at ≥ 2500 µg/plate and in tester strains WP2P (WP2 pKM101) and WP2 uvrA at 5000 µg/plate in the absence of S9 mix and TA 1537 and TA 98 with S9 at 5000 µg/plate and in the second experiment for tester strains TA 1535, TA 1537, TA 98, TA 100 and WP2P with and without S9 mix and in tester strain WP2 uvrA in the absence of S9 mix at 5000 µg/plate .

C. SOLUBILITY

There was no precipitation of the test substance reported.

D. MUTATION ASSAY

Although a statistically significant increase in the mean number of revertant colonies was obtained in tester strains TA 100 and WP2P (WP2 pKM101) at 500 µg/plate in the presence and absence of S9 mix, respectively, and for strains TA 98 and WP2P at 500 µg/plate in the absence of S9 mix, there was no dose response relationship and the observations were not consistent in individual experiments.

The number of revertants induced by the test item were comparable to those of the corresponding solvent controls. The positive control compounds induced the expected results, indicating the functionality of the S9 mix and demonstrating the validity of the test system.

Table B.6.4.1.13-1: Glyphosate Acid: An Evaluation of Mutagenic Potential Using *S. Typhimurium* and *E. Coli*. ([redacted] 1996), first experiment, plate-incorporation test

Experiment 1: Standard plate test (SPT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2P** (WP2 pKM101)		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control												
DMSO mean	10.2	10.2	2.8	3.2	23.8	23.4	69.8	80	36.7	45.8	121.6	154
± SD	± 3.2	± 4.3	± 0.8	± 1.6	± 5.2	± 4.8	± 4.3	± 5.5	± 7.1	± 3.5	± 5.2	± 8.2
Test item [µg/plate]												
100 mean	9.7	12.3	2.3	2.0	23.0	25.3	69.7	79.0	34.0	44.0	127.3	150.0
± SD	± 1.2	± 6.5	± 1.5	± 1.0	± 6.2	± 6.1	± 3.8	± 9.0	± 5.3	± 6.6	± 10.6	± 11.8
200 mean	8.3	14.0	2.0	3.3	23.0	23.7	68.0	80.3	39.3	51.0	111.0	162.3
± SD	± 4.0	± 3.6	± 0.0	± 1.5	± 1.7	± 2.3	± 3.5	± 8.5	± 2.1	± 7.0	± 9.2	± 14.6
500 mean	11.0	11.7	2.3	2.3	20.7	25.0	67.3	**94.7****	32.7	**54.0***	124.3	162.0
± SD	± 1.7	± 3.8	± 1.2	± 1.5	± 4.0	± 5.3	± 2.1	**± 1.5**	± 3.5	**± 5.2**	± 21.5	± 5.6
1000 mean	7.0	13.3	2.3	3.3	24.0	25.0	60.0	89.3	36.7	43.7	133.7	139.7
± SD	± 1.7	± 2.3	± 2.3	± 0.6	± 2.0	± 3.5	± 1.7	± 13.3	± 4.6	± 4.0	± 17.0	± 26.4
2500 mean	4.7	8.7	1.7	3.0	10.7	20.3	19.3	72.0	26.3	44.0	102.7	151.3
± SD	± 2.9	± 2.5	± 1.2	± 2.6	± 6.0	± 6.7	± 7.4	± 3.6	± 1.2	± 2.6	± 16.2	6.7
5000 mean	1.0	7.0	1.7	1.0	2.7	7.0	1.7	51.7	13.7	23.7	64.0	134.0
± SD	± 1.0	± 3.5	± 0.6	± 1.0	± 2.5	± 5.3	± 2.1	± 13.7	± 4.5	± 3.5	± 4.0	± 18.3
Positive control												

Table B.6.4.1.13-1: Glyphosate Acid: An Evaluation of Mutagenic Potential Using *S. Typhimurium* and *E. Coli*. ([redacted], 1996), first experiment, plate-incorporation test

Experiment 1: Standard plate test (SPT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2P** (WP2 pKM101)		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[§] 1. mean	114.0**	80.0**	14.0**	20.5**	160.5**	114.5**	245.5**	207.0**	103.5**	84.5**	405.0**	296.5**
± SD	± 18.4	± 11.3	± 5.7	± 2.1	± 14.8	± 0.7	± 49	± 12.7	± 0.7	± 10.6	± 9.9	± 13.4
[§] 2. mean	234.0**	99.0**	27.0**	36.0**	384.5**	287.0**	727.0**	552.5**	149.0**	127.5**	2004.0**	1050.0**
± SD	± 1.4	± 21.2	± 9.9	± 8.5	± 0.7	± 2.8	± 0.0	± 60.1	± 8.5	± 10.6	± 96.2	± 118.8
[§] 3. mean	752.5**	191.0**	63.0**	70.0**	734.5**	361.5**	1165.5**	1037.5**	182.5**	163.0**	2737.0**	2349.5**
± SD	± 111.0	± 12.7	± 7.1	± 0.0	± 101.1	± 6.4	± 40.3	± 30.4	± 3.5	± 12.7	± 315.4	± 109.6

[§] Information on respective positive control is reported in Material and Method section I.A.2. 3 Concentrations were used per plate, 1 = low, 2 = middle, 3 High.

* P < 0.05;
** P < 0.01 (One sided t-Test assumes Test > Control)

Table B.6.4.1.13-2: Glyphosate Acid: An Evaluation of Mutagenic Potential Using *S. Typhimurium* and *E. Coli*. ([redacted], 1996), second experiment, pre-incubation test

Experiment 2: Pre-incubation test (PIT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2P** (WP2 pKM101)		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control												
DMSO mean	9.2	11.4	3.0	3.8	18.2	24.2	81.8	83.0	37.4	54.2	98.8	134.6
± SD	± 3.1	± 0.9	± 1.2	± 1.3	± 2.6	± 7.7	± 10.1	± 6.6	± 2.9	± 4.9	± 10.4	± 15.5
Test item												
100 mean	10.3	9.7	3.7	5.0	19.0	18.7	80.0	87.7	34.0	56.0	99.0	150.7
± SD	± 3.2	± 0.6	± 1.5	± 1.0	± 2.0	± 2.1	± 3.6	± 2.5	± 1.0	± 2.0	± 9.5	± 19.6
200 mean	8.3	10.7	3.3	4.7	20.3	22.7	92.3	81.0	40.0	52.7	97.0	135.0 [C]
± SD	± 4.0	± 3.1	± 1.2	± 1.5	± 2.1	± 2.1	± 4.0	± 7.5	± 3.6	± 8.1	± 15.1	± 15.6
500 mean	9.0	10.7	3.3	4.7	**23.0***	22.3	71.3	82.0	**42.3***	54.7	94.7	140.0
± SD	± 1.0	± 0.6	± 1.5	± 0.6	**± 2.6**	± 2.9	± 3.2	± 6.2	**± 4.0**	± 5.1	± 1.5	± 16.4
1000 mean	7.7	10.0	3.0	3.3	20.0	19.3	75.0	85.3	38.3	51.7	101.0	134.0
± SD	± 1.5	± 1.7	± 1.0	± 0.6	± 5.3	± 2.9	± 6.6	± 3.2	± 2.3	± 2.5	± 6.0	± 6.2
2500 mean	7.7	8.0	2.7	4.0	13.0	11.3	63.3	56.3	24.0	43.3	88.7	134.3
± SD	± 3.8	± 1.7	± 0.6	± 1.0	± 1.7	± 7.4	± 5.0	± 16.2	± 2.6	± 7.4	± 7.5	± 18.8

Table B.6.4.1.13-2: Glyphosate Acid: An Evaluation of Mutagenic Potential Using *S. Typhimurium* and *E. Coli*. ([redacted], 1996), second experiment, pre-incubation test

Experiment 2: Pre-incubation test (PIT)												
Strain	**TA 1535**		**TA 1537**		**TA 98**		**TA 100**		**WP2P** (WP2 pKM101)		**WP2 uvrA**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
5000 mean	3.3	5.0	0.7	2.3	5.7	4.0	22.3	28.0	14.7	23.7	39.7	92.7
± SD	± 2.5	± 2.6	± 1.2	± 0.6	± 5.5	± 0.0	± 2.3	± 10.8	± 3.8	± 10.6	± 11.6	± 17.8
Positive control												
[§] 1. mean	148.5**	36.0**	10.5**	13.0**	69.0**	85.0**	232.0**	163.0**	88.0**	104.0**	210.0**	368.5**
± SD	± 4.9	± 12.7	± 7.8	± 7.1	± 7.1	± 22.6	± 2.8	± 56.6	± 11.3	± 18.4	± 11.3	± 37.5
[§] 2. mean	275.0**	92.5**	33.0**	25.5**	258.0**	219.5**	418.5**	327.0**	140.5**	128.5**	377.5**	1337.0**
± SD	± 5.7	± 4.9	± 11.3	± 10.6	± 11.3	± 27.6	± 30.4	± 9.9	± 17.7	± 0.7	± 33.2	± 328.1
[§] 3. mean	786.0**	143.0**	63.5**	50.0**	927.0**	422.0**	1150.0**	759.5**	180.5**	143.5**	1460.5**	2170.0**
± SD	± 28.3	± 2.8	± 2.1	± 5.7	± 25.5	± 108.9	± 108.9	± 201.5	± 3.5	± 3.5	± 159.1	± 48.1
[§] Information on respective positive control is reported in Material and Method section I.A.2. 3 Concentrations were used per plate, 1 = low, 2 = middle, 3 High.												
C = 1 of 3 plates was contaminated												
* P < 0.05 ** P < 0.01 (One sided t-Test assumes Test > Control)												

III. CONCLUSION:

In conclusion, under the experimental conditions reported, the test item is not mutagenic in the Ames test (standard plate and pre-incubation method) with and without metabolic activation.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2P and WP2 uvrA) with and without metabolic activation.

The study was performed according to OECD guideline 471 (1997) and compliant with GLP. There were only minor deviations from the guideline, which were considered to not compromise the outcome of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:

It is agreed with the applicant's conclusion. Glyphosate acid (batch: P24, purity: 95.6%) was negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *E. coli* WP2P (WP2 pKM101) and WP2 uvrA) in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015).

B.6.4.1.14. *In vitro studies – bacterial gene mutation, study 14*

Data point	CA 5.4.1/014
Report author	[redacted]

Report year	1996
Report title	Technical glyphosate: Reverse mutation assay "Ames test" using *Salmonella typhimurium* and *Escherichia coli*
Report No	434/014
Document No	Not reported
Guidelines followed in study	OECD 471 (1983), Commission Directive (EC) 92/69/EEC Method B14 (1992), US EPA (TSCA) guidelines
Deviations from current test guideline OECD 471 (1997)	2-Aminoanthracene was used as sole positive control substance demonstrating the functionality of the S9 mix. It was reported that cytotoxicity was observed, but it was not indicated for which strain and for which concentration. Historical control data on vehicle and positive controls were not provided. In the repeat-experiment, no parameter was changed. Both experiments were conducted under the same conditions using the plate incorporation method.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the deviations noted above.

2. Full summary

Technical glyphosate (batch: H95D161A, purity 95.3%) was investigated in the Ames standard plate test (plate-incorporation method) for its potential to induce gene mutations in bacteria. *S. typhimurium* strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2uvrA were exposed to the test item in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction) for approximately 48 hours at 37 °C. Vehicle (distilled water) and positive controls were included in each experiment. Based on the results of a preliminary toxicity test in strains TA100 and WP2uvrA, the concentrations for the main mutagenicity study were selected.

In two independent experiments, the cells were exposed to test item concentrations in the range of 50 to 5000 µg/plate. After 48 hours of incubation, the plates were scored for revertant colonies and examined for a thinning of the bacterial background lawn.

There was no precipitation of the test item observed. Cytotoxicity was reported, but detailed observations on cytotoxicity of individual strains and concentrations were not specified in the study report. Cytotoxicity was evaluated retrospectively by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding vehicle control. A cytotoxic effect was evident when the number of revertant colonies was 50% of the value in corresponding vehicle controls. In the main mutagenicity test, cytotoxicity evident as a decrease in the frequency of revertant colonies was observed in the second experiment only at 5000 µg/plate, in tester strains TA98, TA100 and TA1535 in the absence of S9 mix and in tester strain TA1535 in the presence of S9 mix.

There was no significant increase in the frequency of revertant colonies as compared to vehicle controls recorded for any of the five bacteria strains at any dose level, either with or without metabolic activation.

The number of revertants induced by the vehicle control was within the range of spontaneous revertant rates for each strain. All of the positive control chemicals produced marked increases in the frequency of revertant colonies, demonstrating the activity of the S9 mix.

Based on the experimental results and under the experimental conditions of the present study, technical glyphosate is negative for gene mutation in bacteria in the Ames standard plate test (plate incorporation method) in the presence and absence of metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Technical Glyphosate

Identification: /

Description: White powder,

Lot/Batch #: H95D161A

Purity:	95.3 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) and the stability of the test item in vehicle was not stated in the study report (at the responsibility of the sponsor).
Solvent (vehicle) used:	Sterile distilled water

2. Control materials:

Negative control:	Controls which remained untreated were included in each experiment.
Solvent (vehicle) control:	Sterile distilled water
Solvent (vehicle) /final concentration:	0.1 mL per plate
Positive controls:	Please refer to the table below.

Strain	**Metabolic activation**	**Mutagen**	**Conc. [µg/plate]**
S. typhimurium strains			
TA 100	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	3.0
	+S9	2-Aminoanthracene (2AA)	1.0
TA 1535	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	5.0
	+S9	2-Aminoanthracene (2AA)	2.0
TA 98	-S9	4-Nitroquinoline-1-oxide (4NQO)	0.2
	+S9	2-Aminoanthracene (2AA)	0.5
TA 1537	-S9	9-Aminoacridine (9AA)	80
	+S9	2-Aminoanthracene (2AA)	2.0
E. coli strain			
WP2 uvrA	-S9	N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	2.0
	+S9	2-Aminoanthracene (2AA)	10.0

3. Metabolic activation:

S9 mix was obtained from the livers of male Sprague-Dawley rats, weighing approx. 200 g. The animals received a single intraperitoneal injection of Aroclor 1254 at 500 mg/kg bw. The rats were sacrificed 5 days after administration and the liver homogenate was prepared. Prior to the experiment, the S9 mix was prepared immediately by mixing S9 fraction and co-factor.

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay

Plate incorporation test - S9 mix:	
Concentrations:	50, 150, 500, 1500 and 5000 µg/plate
Tester strain:	TA 100 and WP2 uvrA
Replicates:	Duplicate

(b) Experiment 1 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	50, 150, 500, 1500 and 5000 µg/plate
Tester strain:	TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA
Replicates:	Triplicate

(c) Experiment 2 of the main mutation assay

Plate incorporation test ± S9 mix:	
Concentrations:	50, 150, 500, 1500 and 5000 µg/plate
Tester strains:	TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA
Replicates:	Triplicate

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 19 Aug – 13 Nov 1995
   Finalisation date: 20 Feb 1996

2. **Standard plate test (plate-incorporation test, SPT):**
An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of S9 phosphate buffer (in tests without metabolic activation) were added to 2.0 mL of molten, histidine/tryptophan supplemented medium. All compounds were mixed and equally distributed onto the surface of sterile Vogel-Bonner minimal agar plates. After approximately 48 hours of incubation at 37 °C, the plates were scored for revertant colonies and examined for a diminution of the bacterial background lawn.

3. **Cytotoxicity**
Toxicity was detected by
- a reduction in the number of spontaneous revertant colonies
- a clearing or diminution of the background lawn (= reduced his^- and trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

4. **Statistics**
The test results were analysed for statistical significance according to Kirkland (1989)[2].

5. **Acceptance criteria**
Acceptance criteria were not defined in the study report. Prior to use, the bacteria strains where checked for characteristics, viability and spontaneous reversion rates.

6. **Evaluation criteria**
A test item was considered to show a positive response if it induced a dose-related, statistically significant increase in the mutation rate of at least twice the spontaneous reversion rate in one or more strains in the presence and/or absence of metabolic activation in both experiments at sub-toxic levels.
A test item was considered to show a negative response if the number of induced revertants compared to spontaneous revertants were less than two-fold at each dose level, the intervals of which should be between 2 and 5 fold and extent to the limits imposed by toxicity, solubility or up to the maximum recommended dose of 5000 µg/plate.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

[2] Kirkland, D.J., UKEMS Sub-committee on Guidelines for Mutagenicity Testing. Report Part III (1989), Cambridge University Press.

B. CYTOTOXICITY

Although cytotoxicity was noted in the preliminary toxicity study and in the main mutagenicity study, detailed observations on cytotoxicity of individual strains and concentrations were not further specified in the study report. Cytotoxicity was evaluated retrospectively by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding vehicle control. A cytotoxic effect was evident when the number of revertant colonies was 50 % or less of the value in corresponding vehicle controls.

In the preliminary toxicity test (-S9 mix), cytotoxicity was observed in strain TA 100 at 5000 µg/plate.

In the main mutagenicity test, cytotoxicity evident as a decrease in the frequency of revertant colonies was observed in the second experiment only at 5000 µg/plate, in tester strains TA 98 and TA 100 in the absence of S9 mix and in tester strain TA 1535 in the presence of S9 mix.

C. SOLUBILITY

Precipitation of the tests item was not reported.

D. MUTATION ASSAY

There was no significant increase in the frequency of revertant colonies as compared to vehicle controls recorded for any of the five bacteria strains at any dose level, either with or without metabolic activation.

The number of revertants induced by the vehicle control was within the range of spontaneous revertant rates for each strain. All of the positive control chemicals produced marked increases in the frequency of revertant colonies, demonstrating the activity of the S9 mix.

Table B.6.4.1.14-1: Technical glyphosate: Reverse mutation assay "Ames test" using Salmonella typhimurium and Escherichia coli ([redacted] 1996), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA100**		**TA1535**		**WP2uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated (data obtained in the range finding study)										
mean	74	/	8	/	11	/	11	/	6	/
± SD	± 15.3		± 1.2		± 0.6		± 1.2		± 1.5	
Vehicle control										
Water mean	128	116	13	12	18	18	20	36	8	8
± SD	± 11.5	± 8.2	± 2.5	± 3.8	± 6.7	± 6.7	± 5.7	± 10.8	± 2.3	± 4.4
Test item [µg/plate]										
50 mean	124	105	17	10	13	15	25	32	11	9
± SD	± 16.8	± 12.9	± 2.6	± 2.0	± 2.5	± 3.1	± 8.2	± 3.1	± 5.0	± 3.6
150 mean	106	101	13	10	20	18	17	29	6	12
± SD	± 19.2	± 15.6	± 4.6	± 4.2	± 3.2	± 4.0	± 3.8	± 2.6	± 3.1	± 2.9
500 mean	121	118	15	11	16	18	19	29	12	11
± SD	± 3.8	± 13.5	± 4.0	± 1.0	± 2.5	± 3.1	± 3.1	± 9.0	± 2.1	± 4.0
1500 mean	106	93	13	11	19	20	21	36	10	10
± SD	± 12.9	± 10.2	± 6.7	± 3.1	± 6.7	± 3.1	± 9.9	± 8.1	± 3.1	± 2.6
5000 mean	109	116	13	10	22	22	17	36	9	11
± SD	± 20.2	± 18.7	± 2.0	± 0.6	± 4.0	± 5.5	± 9.6	± 8.6	± 2.6	± 2.3
Positive control										
§ mean	307	406	587	110	1926	616	164	981	263	148
± SD	± 6.0	± 69.2	± 222.4	± 2.3	± 96.0	± 111.2	± 18.5	± 93.6	± 86.5	± 21.5

Table B.6.4.1.14-1: Technical glyphosate: Reverse mutation assay "Ames test" using Salmonella typhimurium and Escherichia coli ([redacted], 1996), first experiment										
Experiment 1: Standard plate test (SPT)										
Strain	**TA100**		**TA1535**		**WP2uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
[§] Information on respective positive control is reported in Material and Method section I.A.2										

Table B.6.4.1.14-2: Technical glyphosate: Reverse mutation assay "Ames test" using *Salmonella typhimurium* and *Escherichia coli* [redacted], 1996), second experiment										
Experiment 2: Standard plate test (SPT)										
Strain	**TA100**		**TA1535**		**WP2uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Untreated (reported as spontaneous mutation rates (main study) in the study report)										
mean	123	/	16	/	20	/	26	/	11	/
± SD	± 16.6		± 3.2		± 0.6		± 3.8		± 5.6	
Vehicle control										
DMSO mean	156	149	38	17	29	27	32	41	16	11
± SD	± 10.7	± 17.0	± 7.0	± 5.3	± 8.0	± 3.2	± 5.0	± 11.9	± 1.0	± 2.5
Test item [µg/plate]										
50 mean	166	142	42	13	25	25	34	31	14	7
± SD	± 1.5	± 13.3	± 1.2	± 4.6	± 1.7	± 2.6	± 5.9	± 6.7	± 2.6	± 1.0
150 mean	165	141	32	13	18	25	31	33	11	13
± SD	± 2.5	± 8.7	± 4.0	± 4.0	± 3.2	± 2.3	± 6.5	± 4.2	± 4.5	± 5.6
500 mean	152	121	33	13	43	23	28	35	10	11
± SD	± 1.5	± 15.3	± 5.8	± 3.1	± 37.3	± 5.0	± 7.0	± 4.5	± 5.1	± 2.1
1500 mean	147	109	27	14	13	22	33	28	12	11
± SD	± 5.9	± 5.1	± 7.0	± 5.1	± 1.7	± 2.3	± 8.1	± 2.6	± 3.5	± 1.7
5000 mean	78	107	30	8	19	15	13	32	8	7
± SD	± 9.5	± 23.7	± 0.6	± 1.5	± 2.9	± 1.0	± 7.2	± 7.0	± 1.5	± 3.5
Positive control										
[§] mean	725	585	482	110	991	248	183	287	865	222
± SD	± 34.1	± 28.0	± 14.2	± 1.5	± 15.0	± 23.7	± 50.8	± 43.5	± 137.5	± 28.3
[§] Information on respective positive control is reported in Material and Method section I.A.2										

III. CONCLUSION:

Based on the results and under the experimental conditions of the present study, technical glyphosate is negative for gene mutation in bacteria in the Ames standard plate test (plate incorporation method) in the presence and absence of metabolic activation.

Assessment and conclusion by applicant:
Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537 and *E. coli* WP2 uvrA) with and without metabolic activation.

The study was conducted according to OECD guideline 471 (1983) and in compliance with GLP. There were only minor deviations when compared to the currently valid OECD guideline 471 (1997), which were considered to not compromise the validity of the study. The study was considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Technical glyphosate was negative for gene mutation in bacteria (S. typhimurium TA 100, TA 98, TA 1535 and TA 1537 and E. coli WP2 uvrA in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015). In contrast to the applicant and the previous evaluation, however, the study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.15. *In vitro studies – bacterial gene mutation, study 15*

Data point	CA 5.4.1/015
Report author	[redacted]
Report year	1995
Report title	HR-001: Reverse Mutation Test
Report No	IET 94-0142
Document No	Not reported
Guidelines followed in study	U.S. EPA FIFRA Guidelines, Subdivision F; similar to OECD guideline 471 (1997)
Deviations from current test guideline OECD 471 (1997)	Compared to OECD 471 (1997) information on historical control data were not reported. In the repeat-experiment, no parameter was changed. The deviation is not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the deviations noted above.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *E. coli* strain WP2 uvrA were exposed to glyphosate technical (HR-001, batch: 940908-1, purity: 95.68 %) in the presence and absence of metabolic activation (phenobarbital and 5,6 benzoflavone-induced rat liver S9 fraction). The test was performed for 48 hours at 37 °C using the pre-incubation method. Vehicle (sterile water) and positive controls were included in each experiment. The concentrations were selected based on a preliminary dose-range finding test in which the test item did not show cytotoxicity to any strain up to the highest dose of 5000 µg/plate tested. In two independent experiments of the main mutagenicity test, the test item was tested according to the pre-incubation method in triplicates of five concentrations ranging from 156 to 5000 µg/plate.

Precipitation of the test substance was not reported, although test item formulations were used at concentrations exceeding the mentioned range of solubility of the solvent. A relevant increase in the number of his^+ and trp^+ revertants (exceeding a factor of 2 when compared to solvent controls) was not observed in any experiment either with or without S9 mix. Vehicle controls showed low spontaneous reversion rates with and without metabolic activation. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system and the metabolic activity of the S9 mix employed.

Based on the results of the present study, glyphosate is not mutagenic in the Ames pre-incubation test with and without metabolic activation under the experimental conditions chosen.

I. MATERIALS AND METHODS

A: MATERIALS

1. **Test material:** Glyphosate technical

Identification: HR-001

Description: White crystals

Lot/Batch #: 940908-1

Purity: 95.68 %

Stability of test compound: The stability of the test item under storage conditions (at approx. 5 °C in a dark cold room) and the stability of the test item in vehicle were not specified.

Solvent used: Sterile water

2. Control materials:

Negative control: Not specified

Solvent control: Sterile water

Solvent/final concentration: The test substance was applied at concentrations > 12 mg/mL using 0.1 mL per plate.

Positive controls: Please refer to the table below.

Strain	Metabolic activation	Mutagen	Solvent	Conc. [µg/plate]
S. typhimurium strains				
TA 100	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	DMSO	0.01
	+S9	2-Aminoanthracene	DMSO	1.0
TA 1535	-S9	Sodium azide (NaN_3)	Water	0.5
	+S9	2-Aminoanthracene	DMSO	2.0
TA 98	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	DMSO	0.1
	+S9	2-Aminoanthracene	DMSO	0.5
TA 1537	-S9	9-Aminoacridine (9-AA)	Water	80
	+S9	2-Aminoanthracene	DMSO	2.0
E. coli strain				
WP2 uvrA	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	DMSO	0.01
	+S9	2-Aminoanthracene	DMSO	10.0

3. Metabolic activation:

S9 mix was purchased from [redacted] Lot no. RAA-316). The homogenate was produced from the livers of male Sprague-Dawley rats, weighing 192 – 229 g, that received intraperitoneal injections of phenobarbital (30 mg/kg bw on Day 1, each 60 m g/kg bw on Days 2, 3 and 4) and 5,6 benzoflavone on Day 3. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factor.

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADH	4	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA	✓	deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 102					
TA 1538				Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay:

Pre-incubation assay ± S9 mix:	
Concentrations:	*0, 200, 500, 1000, 2000 and 5000 µg/plate*
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	*A single plate was used per condition.*

(b) Mutation assays:

Pre-incubation assay ± S9 mix:	
Concentrations:	*0, 156, 313, 625, 1250, 2500 and 5000 µg/plate*
Tester strains:	TA 1535, TA 1537, TA 98, TA 100 and WP2 uvrA
Replicates:	*Triplicates in two independent experiments*

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 21 Feb – 09 Mar 1995
Finalisation date: 03 Apr 1995

2. Pre-incubation test (PIT):
0.1 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation) or 0.5 mL of 100 mM sodium phosphate buffer (in tests without metabolic activation) were pre-incubated for 20 minutes at 37 °C using a shaker. Subsequently 2 mL of aminoacid-supplemented molten agar was added to each test tube. The contents were mixed uniformly and overlaid on the minimal glucose agar plate. Each concentration and the controls were tested in triplicates. After an incubation period of 48 h at 37 °C, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

3. Cytotoxicity
Cytotoxicity was investigated in a preliminary dose-range finding study. Toxicity was detected by a

- decrease in the number of revertants
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

4. Statistics
Results were judged without statistical analysis.

5. Acceptance criteria
The test was valid if

- The culture of the tester strains, the solution of the test substance and S9 mix were free from contamination or other bacteria.
- A normal number of spontaneous revertant colonies was observed for the solvent control.
- An at least 3-fold increase above the solvent control in the mean number of revertants was observed in the positive control.

6. Evaluation criteria
Results were judged positive without statistical analysis when the following criteria were met:

- A two-fold or greater increase above solvent control in the mean number of revertants was observed.
- This increase in the number of revertants was accompanied by a dose-response relationship.
- This increase in the number of revertants was reproducible.

Reproducibility of results was confirmed by two independent experiments.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY
In the preliminary cytotoxicity test, the test substance did not show any cytotoxicity to any strain up to the highest dose of 5000 µg/plate with and without metabolic activation.

In the main mutation study there was also no cytotoxicity observed up to the highest concentration tested.

C. SOLUBILITY
There was no precipitation of the test substance observed.

D. MUTATION ASSAY
A relevant increase in the number of his^+ or trp^+ revertants was not observed in any experiment at any tested concentration either in the presence or absence of metabolic activation.

The number of revertants induced by the vehicle control was within expected range for each strain, thus demonstrating an acceptable experimental performance.

Appropriate positive control compounds induced a marked increase in the number of revertants, demonstrating the validity of the test system and the functionality of the S9 mix.

Table B.66.4.1.15-1: Reverse Mutation Test ([redacted], 1995), first experiment

Experiment 1: Pre-incubation test (PIT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	117	78	12	9	21	21	37	35	3	7
± *SD*	± *6*	± *5*	± *3*	± *0*	± *3*	± *1*	± *11*	± *4*	± *2*	± *5*
Test item [µg/plate]										
156 mean	119	83	11	6	12	19	40	36	3	9
± *SD*	± *9*	± *4*	± *2*	± *4*	± *2*	± *3*	± *10*	± *2*	± *2*	± *3*

Table B.66.4.1.15-1: Reverse Mutation Test ([redacted], 1995), first experiment

Experiment 1: Pre-incubation test (PIT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
313 mean	117	77	11	7	16	19	42	31	4	5
± *SD*	± *12*	± *5*	± *1*	± *2*	± *5*	± *4*	± *6*	± *3*	± *1*	± *3*
625 mean	139	99	9	6	15	19	39	30	2	8
± *SD*	± *8*	± *11*	± *3*	± *4*	± *1*	± *1*	± *10*	± *5*	± *2*	± *3*
1250 mean	125	93	9	6	22	22	43	37	5	6
± *SD*	± *7*	± *17*	± *1*	± *2*	± *4*	± *5*	± *6*	± *4*	± *4*	± *1*
2500 mean	106	73	3	7	15	16	38	39	3	7
± *SD*	± *12*	± *12*	± *1*	± *6*	± *3*	± *2*	± *10*	± *2*	± *2*	± *3*
5000 mean	105	56	4	3	20	16	39	25	2	4
± *SD*	± *11*	± *9*	± *2*	± *2*	± *5*	± *6*	± *7*	± *5*	± *1*	± *2*
Positive control										
[§] mean	510	606	524	392	305	522	621	360	786	75
± *SD*	± *13*	± *70*	± *37*	± *58*	± *28*	± *15*	± *7*	± *41*	± *82*	± *6*

[§] Information on respective positive control is reported in Material and Method section I.A.2

Table B.6.4.1.15-2: Reverse Mutation Test ([redacted], 1995), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	146	123	9	8	16	17	24	37	5	7
± *SD*	± *11*	± *12*	± *1*	± *2*	± *4*	± *8*	± *4*	± *5*	± *4*	± *2*
Test item [µg/plate]										
156 mean	137	112	10	7	18	15	18	32	7	10
± *SD*	± *7*	± *5*	± *3*	± *3*	± *3*	± *1*	± *7*	± *9*	± *4*	± *4*
313 mean	*140*	*125*	*7*	*7*	*19*	*13*	*20*	*29*	*4*	*9*
± *SD*	± *13*	± *12*	± *2*	± *3*	± *2*	± *4*	± *7*	± *6*	± *1*	± *2*
625 mean	*136*	*113*	*8*	*8*	*17*	*20*	*18*	*35*	*3*	*9*
± *SD*	± *22*	± *4*	± *3*	± *1*	± *3*	± *5*	± *4*	± *6*	± *2*	± *4*
1250 mean	*136*	*107*	*7*	*7*	*15*	*14*	*15*	*28*	*3*	*9*
± *SD*	± *12*	± *9*	± *2*	± *3*	± *5*	± *1*	± *7*	± *4*	± *2*	± *1*
2500 mean	144	89	6	7	18	19	10	20	3	8
± *SD*	± *15*	± *10*	± *2*	± *3*	± *4*	± *7*	± *1*	± *6*	± *1*	± *3*
5000 mean	117	67	10	4	14	17	9	17	4	4
± *SD*	± *14*	± *3*	± *4*	± *2*	± *5*	± *4*	± *2*	± *4*	± *3*	± *2*

Table B.6.4.1.15-2: Reverse Mutation Test ([redacted], 1995), second experiment

Experiment 2: Pre-incubation test (PIT)										
Strain	**TA 100**		**TA 1535**		**WP2 uvrA**		**TA 98**		**TA 1537**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Positive control										
§ mean	595	768	527	322	252	605	742	327	909	87
± SD	*± 23*	*± 56*	*± 87*	*± 32*	*± 27*	*± 35*	*± 36*	*± 20*	*± 131*	*± 9*

§ Information on respective positive control is reported in Material and Method section I.A.2

III. CONCLUSION:

According to the results of the present study, the test item is not mutagenic in the Ames pre-incubation test with and without metabolic activation under the experimental condition of the test.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA) with and without metabolic activation.

The study was conducted according to OECD guideline 471 (1983) and in compliance with GLP. There were only minor deviations when compared to the currently valid OECD guideline 471 (1997), which were considered to not compromise the validity of the study. The study was considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Technical glyphosate was negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 and *E. coli* WP2 uvrA in the presence and absence of metabolic activation under the conditions of this test. This conclusion is in line with the previous evaluation (RAR, 2015). In contrast to the application and the previous evaluation, however, the study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.16. *In vitro studies – bacterial gene mutation, study 16*

Data point:	CA 5.4.1/016
Report author	[redacted]
Report year	1995
Report title	Study of the ability of the test article glyphosate to induce gene mutations in strains of *Salmonella typhimurium*
Report No	940724
Document No	Not reported
Guidelines followed in study	The study was conducted according to the main criteria of OECD TG 471 (1983)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	*S. typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 were exposed to glyphosate (batch NC01; purity not reported) in the presence and absence of metabolic activation (liver S9 fraction) using the plate incorporation assay. Test item concentrations were in the range of 50 – 5000 µg/plate.

Short description of results:	Negative for mutagenicity in bacteria (Ames test) up to limit concentrations of 5000 µg/plate in the presence and absence of metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 4b. **Conclusion AGG:** The study report has been made available to AGG by BVL. The RMS has evaluated the study and agrees with the previous conclusion (RAR, 2015) that the study is not considered reliable for evaluation since the purity of glyphosate was not reported.

B.6.4.1.17. *In vitro studies – bacterial gene mutation, study 17*

Data point:	CA 5.4.1/017
Report author	[redacted]
Report year	1995
Report title	Glyphosate: Reverse mutation assay "Ames test" using *Salmonella typhimurium*
Report No	710/20
Document No	Not reported
Guidelines followed in study	The study was conducted according to the main criteria of OECD TG 471 (1983)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	*S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 were exposed to glyphosate (purity and batch not reported) in the presence and absence of metabolic activation (liver S9 fraction) by the plate incorporation method. Test item concentrations were in the range of 8 to 5000 µg/plate.
Short description of results:	Negative for mutagenicity in bacteria (Ames test) up to limit concentrations of 5000 µg/plate in the presence and absence of metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 4b. **Conclusion AGG:** The study report has been made available to AGG by BVL. The RMS has evaluated the study and agrees with the previous conclusion (RAR, 2015) that the study is not considered acceptable for evaluation since the purity and batch of the test item was not reported and the test was conducted in four valid strains only. Strains like *S. typhimurium* TA 102 or *E. coli* WP2 enabling the detection of cross-linking mutagens were not included.

B.6.4.1.18. *In vitro studies – bacterial gene mutation, study 18*

Data point	CA 5.4.1/018
Report author	[redacted]
Report year	1993
Report title	Mutagenicity – *Salmonella typhimurium* reverse mutation assay (Ames test)
Report No	887-MUT.AMES
Document No	Not reported
Guidelines followed in study	OECD 471 (1983)
Deviations from current test guideline OECD 471 (1997)	The test was conducted in four valid strains only. Strains like *S. typhimurium* TA 102 or *E. coli* WP2 enabling the detection of cross-linking mutagens were not included. In addition, the bacterial cell density was not confirmed and no

	historical control data were provided. Further, acceptance criteria were not specified in the study report.
Previous evaluation	Not accepted in RAR 2015
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be supportive due to the noted deviations.

Glyphosate technical (batch 046, purity: 96.0 %) was assessed for its ability to cause gene mutations in *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Based on the results of a preliminary cytotoxicity test, in which no cytotoxicity was noted up to a concentration of 1000 µg/plate, concentrations for the main mutagenicity assay were selected. Two independent plate-incorporation tests (standard plate tests) were performed, using glyphosate concentrations in the range of 1 – 1000 µg/plate. Solvent (distilled water) and appropriate positive controls were included in each experiment. After 48 hours of incubation at 37 °C, the bacterial background lawn was examined and the number of revertant colonies was counted for each plate.

There was no precipitation up to the highest tested concentration, neither in the presence nor absence of S9 mix. Cytotoxicity, evident as a thinning of the bacterial background lawn, was noted at 1000 µg/plate; however, it was not specified for which strains the observation was made and whether the observation was made in the presence or absence of metabolic activation.

There was no statistically significant, toxicologically relevant increase in the number of revertant colonies observed in any experiment up to the highest concentration, neither in the presence nor in the absence of metabolic activation. Although a statistically significant increase in the number of his^+ revertant colonies of about 40 % was observed for strain TA 100 at the top concentration of 1000 µg/plate in the presence of metabolic activation, the increase was less than two-fold compared to the number of revertant colonies induced by the solvent control and therefore considered to be incidental.

The number of revertant colonies induced by the vehicle and appropriate positive controls were in the expected range, demonstrating the functionality of the S9 mix and the sensitivity of the test system.

Under the conditions of the test, glyphosate technical was negative for gene mutation in bacteria in the presence and absence of metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	FSG 03090 H/05, March 1990
Description:	Solid white coloured crystals, odourless
Lot/Batch #:	046
Purity:	96.0 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature) was guaranteed until the expected expiry date July 1994. The stability of the test item in solvent (vehicle) was not specified.
Solvent (vehicle) used:	Distilled water

2. Control materials:

Negative control:	A negative control was not employed in this study.
Solvent (vehicle) control:	Distilled water
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to table below.

Strain	**Metabolic activation**	**Mutagen**	**Conc. [µg/plate]**
S. typhimurium strains			
TA 100	-S9	Sodium azide	0.5
	+S9	2-Aminofluorene	20.0
TA 1535	-S9	Sodium azide	0.5
	+S9	Sodium azide	0.5
TA 98	+S9	2-Nitrofluorene	2.0
	+S9	2-Aminofluorene	20.0
TA 1537	-S9	9-Aminoacridine	50.0
	+S9	9-Aminoacridine	50.0
TA 1538	-S9	2-Nitrofluorene	5.0
	+S9	2-Aminofluorene	20.0

3. Metabolic activation:

S9 mix was produced from the livers of male Wistar rats, that received a single intraperitoneal injection of Aroclor 1254 at a dose level of 500 mg/kg bw. The livers were prepared five days after the injection. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	**Concentration**	**Unit**
Phosphate buffer (pH 7.4)		
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate, K salt	5	mM
NADP, di-Na salt	4	mM
$MgCl_2$	8	mM
S9	10	% (v/v)

4. Test organisms:

Tester strains				**Bacteria batch checked for**	
S. typhimurium		***E.coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 1538	✓			Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay:

Plate incorporation test ± S9 mix:	
Concentrations:	*10, 30, 100, 350 and 1000 µg/plate*
Tester strains:	TA 100
Replicates:	*Duplicates*

(b) Mutation assays:

Plate incorporation test ± S9 mix:	
Concentrations:	*1, 3, 10, 100 and 1000 µg/plate*
Tester strains:	TA 98, TA 100, TA 1535, TA 1537 and TA 1538
Replicates:	*Triplicates*

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: Dec 1992 – Jan 1993
Finalisation date: 30 Apr 1993

2. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.1 mL test solution, vehicle or positive control, an aliquot of 0.1 mL bacterial culture (2×10^9 cells/mL) and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of 0.05 M phosphate buffer (in tests without metabolic activation) were added to 2 mL of soft agar (supplemented with 0.5 mM L-histidine + 0.5 mM D-biotin). After vortexing, the mixture was overlaid onto the surface of minimal bottom agar plates. Each concentration and the controls were tested in triplicates. The cells were incubated at 37 °C for 48 hours. Afterwards, the background bacterial lawn was examined and the bacterial colonies were counted.

3. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants
- clearing or diminution of the background lawn (= reduced his^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

4. Statistics
For each triplicate plating, an average and standard deviation were calculated. The results were evaluated for statistical analysis; however, it was not specified in the study report which kind of test was used for statistical evaluation.

5. Acceptance criteria
Acceptance criteria were not specified in the study report.

6. Evaluation criteria
A test item was considered as a possible mutagen if the number of revertant colonies was at least two-fold the number of spontaneous revertants observed for the solvent control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY
In the preliminary toxicity test in strain TA 100, a slight thinning of the bacterial background lawn was observed at 350 and 1000 µg/plate in the presence of metabolic activation. The number of revertant colonies was comparable to those of solvent controls.

In the main mutagenicity assay, cytotoxicity, evident as a slight thinning of the bacterial background lawn, was noted at the highest concentration of 1000 µg/plate. However, it was not specified for which strains the observation was made and whether the observation was made in the presence or absence of metabolic activation. Evaluation of the colony shape and size revealed that upon treatment with glyphosate at ≥ 100 µg/plate rough edged colonies were noticed.

C. SOLUBILITY
Precipitation of the test item in minimal basal agar plates was tested in a preliminary precipitation test. There was no precipitation up to the highest concentration of 1000 µg/plate. In the main mutagenicity assay there was as well no precipitation reported at any of the tested concentration, either in the presence or in absence of S9 mix.

D. MUTATION ASSAY
There was no statistically significant, toxicologically relevant increase in the number of revertant colonies observed in any experiment up to the highest concentration, neither in the presence nor in the absence of metabolic activation. A statistically significant increase in the number of his^+ revertant colonies of about 40 % was observed for strain TA 100 at the top concentration of 1000 µg/plate in the presence of metabolic activation, but as the increase was less than two-fold compared to the number of revertant colonies induced by the solvent control, the observation was considered incidental.

The number of revertant colonies induced by the vehicle and appropriate positive controls were in the expected range, demonstrating the functionality of the S9 mix and the sensitivity of the test system.

Table B.6.4.1.18-1: Study of the ability of the test article glyphosate to induce gene mutations in strains of *Salmonella typhimurium* ([redacted] 1993), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 1538**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	27	19	120	114	11	16	6	12	31	11
± SD	*± 2.0*	*± 2.0*	*± 9.0*	*± 5.1*	*± 4.5*	*± 2.0*	*± 1.7*	*± 2.6*	*± 1.5*	*± 1.0*
Test item [µg/plate]										

Table B.6.4.1.18-1: Study of the ability of the test article glyphosate to induce gene mutations in strains of *Salmonella typhimurium* ([redacted], 1993), first experiment

Experiment 1: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 1538**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
1 mean	34	21	99	124	12	12	10	14	31	10
± *SD*	± *2.5*	± *1.5*	± *2.5*	± *11.1*	± *1.0*	± *2.5*	± *5.7*	± *4.6*	± *2.8*	± *4.1*
3 mean	31	18	109	134	10	18	6	15	33	9
± *SD*	± *0.6*	± *2.0*	± *30.3*	± *9.0*	± *3.2*	± *2.0*	± *0.6*	± *5.0*	± *1.0*	± *3.2*
10 mean	28	31	117	120	10	18	4	9	29	8
± *SD*	± *1.0*	± *3.6*	± *9.2*	± *4.6*	± *3.4*	± *4.9*	± *0.6*	± *2.5*	± *1.2*	± *1.5*
100 mean	32	25	125	154	7	16	7	17	28	11
± *SD*	± *4.5*	± *4.0*	± *30.2*	± *9.6*	± *0.6*	± *4.0*	± *1.7*	± *4.0*	± *2.3*	± *2.0*
1000 mean	31	22	126	**155***	7	15	7	11	28	9
± *SD*	± *5.5*	± *3.8*	± *12.5*	± *14.2*	± *1.1*	± *1.0*	± *2.6*	± *3.5*	± *3.0*	± *3.7*
Positive control										
§ mean	78	89	473	356	190	375	89	74	84	84
± *SD*	± *10.4*	± *3.6*	± *41.6*	± *40.4*	± *10.0*	± *25.0*	± *16.3*	± *8.1*	± *20.1*	± *6.0*

§ Information on respective positive control is reported in Material and Method section A.2

* A statistically significant increase in the number of his$^+$ revertant colonies of about 40 % was observed for strain TA100 at the top concentration of 1000 µg/plate in the presence of metabolic activation (trial 1/2 combined), but as the increase was less than two-fold compared to the number of revertant colonies induced by the solvent control, the observation was considered incidental

Table B.6.4.1.18-2: Study of the ability of the test article glyphosate to induce gene mutations in strains of *Salmonella typhimurium* ([redacted], 1993), second experiment

Experiment 2: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 1538**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control										
Water mean	14	17	91	111	12	9	5	4	26	6
± *SD*	± *4.0*	± *3.4*	± *4.3*	± *18.0*	± *1.7*	± *1.5*	± *2.5*	± *1.1*	± *6.0*	± *2.0*
Test item										
1 mean	12	13	115	103	8	11	2	9	15	8
± *SD*	± *2.5*	± *0.5*	± *32.5*	± *9.8*	± *5.1*	± *4.5*	± *0.5*	± *4.0*	± *3.0*	± *1.5*
3 mean	17	16	114	94	6	10	4	6	14	8
± *SD*	± *5.1*	± *1.0*	± *11.3*	± *8.1*	± *1.5*	± *4.1*	± *1.7*	± *2.0*	± *1.5*	± *2.0*
10 mean	12	13	100	116	3	6	4	9	14	7
± *SD*	± *5.0*	± *2.5*	± *7.5*	± *9.6*	± *1.2*	± *1.5*	± *2.6*	± *3.5*	± *2.5*	± *1.0*
100 mean	15	11	114	101	5	8	4	11	11	8
± *SD*	± *3.6*	± *2.8*	± *13.0*	± *7.5*	± *0.5*	± *2.0*	± *1.1*	± *3.7*	± *4.5*	± *3.2*

Table B.6.4.1.18-2: Study of the ability of the test article glyphosate to induce gene mutations in strains of *Salmonella typhimurium* ([redacted], 1993), second experiment

Experiment 2: Standard plate test (SPT)										
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**TA 1538**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
1000 mean	12	14	85	**177***	6	7	5	7	12	7
± *SD*	± *2.5*	± *3.4*	± *3.0*	± *13.4*	± *2.0*	± *0.5*	± *3.2*	± *1.5*	± *1.5*	± *1.1*
Positive control										
§ mean	95	135	396	420	171	372	85	128	75	76
± *SD*	± *22.9*	± *13.2*	± *15.3*	± *20.0*	± *7.6*	± *7.5*	± *6.4*	± *7.0*	± *12.8*	± *15.0*

§ Information on respective positive control is reported in Material and Method section A.2

* A statistically significant increase in the number of his$^+$ revertant colonies of about 40 % was observed for strain TA100 at the top concentration of 1000 µg/plate in the presence of metabolic activation (trial 1/2 combined), but as the increase was less than two-fold compared to the number of revertant colonies induced by the solvent control, the observation was considered incidental

III. CONCLUSION

Based on the experimental results, glyphosate technical did not induce a positive response in the number of revertant colonies in any experiment in any tester strain and was therefore considered negative for mutagenicity in bacteria, both, in the presence and absence of metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 98, TA 100, TA 1535, TA 1537 and TA1538) with and without metabolic activation.

The study was considered as supporting information as it was conducted in 4 valid strains only. Strains like *S. typhimurium* TA102 or *E. coli* WP2 enabling the detection of cross-linking mutagens were not included. Further deviations were of minor degree and considered to not compromise the validity of the study.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. For TA 100 a significant increase in revertants/plate were found in both assays in the presence of S9. However, as the increase in revertant colonies was less than 2-fold (40% increase compared with vehicle controls) this finding is considered incidental. Therefore, technical glyphosate was considered negative for gene mutation in the valid bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537 in the presence and absence of metabolic activation under the conditions of this test. The study is considered as supportive only due to the noted deviations. In the previous assessment (RAR 2015) this study was considered not acceptable.

B.6.4.1.19. *In vitro studies – bacterial gene mutation, study 19*

Data point:	CA 5.4.1/019
Report author	[redacted]
Report year	1993
Report title	Mutagenicity evaluation of glyphosate in *Salmonella* / microsomal reversion assay (Ames test)
Report No	87BMA012-E
Document No	Not reported

Guidelines followed in study	No guideline followed. The test was conducted similarly to OECD 471 (1997).
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	*S. typhimurium* strains TA 98 and TA 100 were exposed to glyphosate isopropylamine salt (SN-750721, batch: not reported, purity: 64 %) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). A single experiment was performed, using the plate-incorporation method and five replicates per condition. Both bacterial strains were exposed to test item concentrations in the range of 0.01 – 100 µg/plate in the presence and absence of S9 mix. Untreated, solvent (DMSO) and positive controls were included. After 2 days of incubation at 37 °C, the number of revertant colonies per plate was determined. Evaluation of precipitation and cytotoxicity were not included in the study report.
Short description of results:	When evaluating cytotoxicity based on the number of revertant colonies in comparison to those of negative controls, treatment with glyphosate isopropylamine salt revealed no evidence for cytotoxicity. There was a statistically significant increase in the frequency of revertant colonies noted for strain TA 100 at 0.01, 0.1, 1 and 100 µg/plate in the absence of metabolic activation, however, the increases were less than two-fold of those of untreated / solvent controls and without a clear dose-response relationship, therefore the findings were considered to be incidental. There were no statistically significant increases in the number of revertant colonies observed upon treatment with the test item in strain TA98. The number of revertant colonies obtained by the untreated and solvent controls were in the expected range. The positive controls strongly increased the number of revertant colonies of more than twice than those of untreated controls, demonstrating the functionality of the S9 mix and the sensitivity of the test. Based on the experimental results, glyphosate isopropylamine salt was not mutagenic in the Ames plate incorporation test, neither with nor without metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study was considered not acceptable as it was conducted in two strains of *S. typhimurium* only (TA 98 and TA 100), with a test material of only 64 % purity. In addition, it was not clear whether the given purity refers to the contents of glyphosate in the formulation or the salt. A single experiment using 5 replicates per conditions was conducted, but a confirmatory experiment was not included. Test item concentrations up to 100 µg/mL were used, but a justification for the selection of concentrations was not given. In addition, no historical control data were provided. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as described by GRG.

B.6.4.1.20. *In vitro studies – bacterial gene mutation, study 20*

Data point	CA 5.4.1/020
Report author	[redacted]
Report year	1991
Report title	Mutagenicity test: Ames Salmonella Assay with Glyphosate, batch 206-JaK-25-1
Report No	12323
Document No	Not reported
Guidelines followed in study	OECD 471 (1983), US EPA FIFRA 84-2

Deviations from current test guideline OECD 471 (1997)	The test was conducted in 4 valid strains only, strains like *S. typhimurium* TA102 or *E. coli* WP2 enabling the detection of cross-linking mutagens were not included. In addition, 2-aminoanthracene was used as sole positive control substance in the presence of S9 mix and historical control data were not provided. Acceptance and evaluation criteria were not specified in the study report.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be supportive due to the noted deviations.

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537 were exposed to glyphosate (batch: 206-JaK-25-1, purity: 98.6 %) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). A preliminary cytotoxicity test was performed in strain TA98. Cytotoxicity was evident at 1670 and 5000 µg/plate in the absence of S9 mix only, but not in the presence of S9 mix. Based on these observations, the concentrations for the main mutagenicity study were selected. In the main mutation assay two independent experiments were performed, using the plate incorporation (first experiment) and the pre-incubation method (second experiment) with concentrations in the range of 160 - 2500 µg/plate in the absence of S9 mix and concentrations in the range of 310 – 5000 µg/plate in the presence of S9 mix. Negative and positive controls were included in each experiment.

Precipitation of the test item was not reported. Cytotoxicity, evident as a reduction in the mean number of spontaneous revertant colonies, was noted solely for strain TA100 at 2500 µg/plate in the absence of S9 mix in both experiments, at 5000 µg/plate in the first experiment with S9 mix and at 2500 and 5000 µg/plate in the second experiment with S9 mix.

There was no statistically significant increase in the number of his^+ revertant colonies observed in any experiment for any strain at any concentration, neither in the presence nor in the absence of S9 mix. The number of revertant colonies induced by the negative and the positive controls were within the expected range, displaying the sensitivity of the test and the functionality of the metabolic activation system.

Based on the results of the present study and under the experimental conditions chosen, glyphosate is not mutagenic in the Ames test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	Not specified
Description:	White powder
Lot/Batch #:	206-JaK-25-1
Purity:	98.6 %
Stability of test compound:	The stability of tehe test item at storage conditions (at room temperature in the dark) or in solvent (vehicle) was not specified.
Solvent (vehicle) used:	Distilled water

2. Control materials:

Negative control:	Untreated controls were included in each experiment.
Solvent (vehicle) control:	Not examined.
Solvent (vehicle)/final concentration:	0.3 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Conc. [µg/plate]
S. typhimurium strains			
TA 100	-S9	Sodium azide	0.5
	+S9	2-Aminoanthracene	1.25
TA 1535	-S9	Sodium azide	1.0
	+S9	2-Aminoanthracene	1.25
TA 98	-S9	2-Nitrofluorene	0.6
	+S9	2-Aminoanthracene	1.25
TA 1537	-S9	2-Nitrofluorene	0.6
	+S9	2-Aminoanthracene	1.25

3. Metabolic activation:

S9 mix was obtained from livers of Wistar Mol:WIST rats weighing approx. 200 g. The animals received a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg bw. The livers were prepared 5 days after treatment. The S9 mix was thawed prior to each experiment and co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	8	mM
S9 Standard plate test (plate-incorporation) Pre-incubation test	 4 7	 % (v/v) % (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E. coli***			
TA 98	✓	WP2 uvrA		deep rough character (rfa)	✓
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	✓
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	✓
TA 1537	✓				
TA 1538				Histidine auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

(a) Preliminary cytotoxicity assay:

Plate incorporation test ± S9 mix:	
Concentrations:	*560, 1670 and 5000 µg/plate*
Tester strains:	TA 98
Replicates:	*Triplicates*

(b) Mutation assays:

Plate incorporation test ± S9 mix:	

Concentrations: -S9 mix +S9 mix	*160, 310, 630, 1300 and 2500 µg/plate* *310, 630, 1300, 2500 and 5000 µg/plate*	
Tester strains:	TA 1535, TA 1537, TA 98 and TA 100	
Replicates:	*Triplicates*	
Pre-incubation test ± S9 mix:		
Concentrations: -S9 mix +S9 mix	*160, 310, 630, 1300 and 2500 µg/plate* *310, 630, 1300, 2500 and 5000 µg/plate*	
Tester strains:	TA 1535, TA 1537, TA 98 and TA 100	
Replicates:	*Triplicates*	

B: STUDY DESIGN AND METHODS

3. Finalisation date: 10 Sep 1991

4. Standard plate test (plate-incorporation test, SPT):
An aliquot of 0.3 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation only) were added to 2 mL of molten top agar (supplemented with 0.5 mM L-histidine + 0.5 mM biotin). After whirl mixing, the mixture was spread on a Vogel-Bonner agar plate and incubated for 48 – 72 hours at 37 °C. Each concentration and the controls were tested in triplicates. Following incubation, the bacterial background lawn was examined and the number of his^+ revertant colonies was counted.

5. Pre-incubation test (PIT):
0.3 mL of test solution or vehicle/positive control, 0.1 mL pre-cultured bacterial suspension and 0.5 mL of S9 mix (in tests with metabolic activation only) were mixed and pre-incubated in a test tube for 30 minutes at 37 °C under gentle shaking. After pre-incubation, 2.0 mL of top agar was added, the mixture was whirl-mixed and spread on a Vogel-Bonner agar plate. After an incubation period of 48 – 72 hours at 37 °C the bacterial background lawn was examined and the number of his^+ revertant colonies was counted.

6. Cytotoxicity
Toxicity was detected by a

- reduction in the number of spontaneous revertants
- clearing or diminution of the background lawn (= reduced his^- or trp^- background growth)

and recorded for all test groups both with and without S9 mix in all experiments.

7. Statistics
Statistical analysis of the negative control versus test data was performed using the Analysis of Variance method. Statistical analysis of the negative versus positive control data was performed using the Student's t-test.

8. Acceptance criteria
Acceptance criteria were not specified in the study report.

9. Evaluation criteria
Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations have not been performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary toxicity test in strain TA98, cytotoxicity was observed in the absence of S9 mix at 1670 and 5000 µg/plate. There was no cytotoxicity noted in the presence of S9 mix. Based on these findings, the top concentrations for the main mutagenicity test were chosen to be 2500 µg/plate without S9 mix and 5000 µg/plate with S9 mix.

In the main mutagenicity assay, no clear depression of the bacterial background growth was observed at any concentration with or without S9 mix. However, cytotoxicity evident as a reduction in the mean number of spontaneous revertants was observed for strain TA100 in both experiments at 2500 µg/plate in the absence of S9 mix, at 5000 µg/plate in the first experiment (standard plate test, plate incorporation method) with S9 mix and at 2500 and 5000 µg/plate in the second experiment (pre-incubation test) with S9 mix. There was no relevant cytotoxicity observed in any other tester strain in the presence or absence of metabolic activation.

C. SOLUBILITY

Precipitation was not evaluated in the study report.

D. MUTATION ASSAY

There was no statistically significant increase in the number of his^+ revertant colonies observed in any experiment for any strain at any concentration, neither in the presence nor in the absence of S9 mix. The number of revertant colonies induced by the negative and the positive controls were within the expected range, displaying the sensitivity of the test and the functionality of the metabolic activation system.

Table B.6.4.1.20-1: Mutagenicity test: Ames Salmonella Assay with Glyphosate ([redacted] 1991), first experiment

Experiment 1: Standard plate test (SPT)								
Strain	**TA100**		**TA 98**		**TA 1537**		**TA 1535**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Negative control								
mean	175.7	198.3	33.0	36.0	11.0	13.0	20.0	20.7
± *SD*	± *14.4*	± *10.4*	± *12.5*	± *4.4*	± *1.0*	± *1.0*	± *1.0*	± *2.9*
Test item [µg/plate]								
160 mean	153.0	/	30.3	/	10.7	/	23.0	/
± *SD*	± *5.3*	/	± *0.6*	/	± *3.2*	/	± *0.0*	/
310 mean	182.3	182.3	33.0	38.7	11.0	10.3	19.7	25.7
± *SD*	± *11.5*	± *27.5*	± *5.6*	± *7.7*	± *1.7*	± *3.2*	± *5.0*	± *4.0*
630 mean	151.3	187.7	30.3	42.7	10.0	12.3	19.3	20.0
± *SD*	± *14.7*	± *20.4*	± *4.5*	± *5.5*	± *1.0*	± *2.1*	± *5.1*	± *3.0*
1300 mean	97.7	124.3	27.3	35.0	10.3	11.7	13.3	21.3
± *SD*	± *7.8*	± *10.0*	± *5.7*	± *6.6*	± *2.3*	± *2.1*	± *1.5*	± *4.9*
2500 mean	75.7[#]	104.0	23.0	35.7	9.3	13.0	13.0	16.3
± *SD*	± *18.6*	± *8.7*	± *2.6*	± *5.7*	± *0.6*	± *5.0*	± *1.0*	± *0.6*
5000 mean	/	76.3[#]	/	26.0	/	8.7	/	13.3

Table B.6.4.1.20-1: Mutagenicity test: Ames Salmonella Assay with Glyphosate ([redacted] 1991), first experiment

Experiment 1: Standard plate test (SPT)								
Strain	**TA100**		**TA 98**		**TA 1537**		**TA 1535**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
± SD	*/*	*± 12.6*	*/*	*± 3.5*	*/*	*± 0.5*	*/*	*± 3.5*
Positive control								
§ mean	880.0**	1120.0**	317.3**	1076.7**	127.7**	147.3**	1053.3**	218.7**
± SD	*± 98.5*	*± 158.2*	*± 12.1*	*± 326.5*	*± 22.1*	*± 6.4*	*± 225.0*	*± 16.7*

§ Information on respective positive control is reported in Material and Method section I.A.2

** Statistically significant at 1 % level (analysis of variance)

Cytotoxicity observed

Table B.6.4.1.20-2: Mutagenicity test: Ames Salmonella Assay with Glyphosate ([redacted], 1991), second experiment

Experiment 2: Pre-incubation Test (PIT)								
Strain	**TA100**		**TA 98**		**TA 1537**		**TA 1535**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Negative control								
mean	182.3	197.0	44.3	20.3	13.7	5.3	26.3	14.7
± SD	*± 9.5*	*± 18.2*	*± 7.5*	*± 1.5*	*± 2.1*	*± 0.6*	*± 4.9*	*± 0.6*
Test item [µg/plate]								
160 mean	195.7	/	52.0	/	15.7	/	28.7	/
± SD	*± 6.8*	*/*	*± 4.0*	*/*	*± 1.5*	*/*	*± 4.5*	*/*
310 mean	185.0	182.7	49.3	23.3	14.0	7.3	30.3	15.7
± SD	*± 19.1*	*± 18.8*	*± 11.9*	*± 7.5*	*± 6.1*	*± 2.1*	*± 3.8*	*± 2.1*
630 mean	133.3	169.7	44.0	22.3	8.3	6.0	32.7	15.0
± SD	*± 10.5*	*± 8.0*	*± 9.2*	*± 0.6*	*± 3.1*	*± 1.0*	*± 7.5*	*± 1.0*
1300 mean	110.0	122.0	35.0	25.0	16.3	6.0	29.3	14.3
± SD	*± 14.0*	*± 4.0*	*± 2.6*	*± 2.6*	*± 2.9*	*± 1.0*	*± 3.1*	*± 0.6*
2500 mean	54.3#	94.0#	31.7	19.7	11.3	6.0	20.7	15.0
± SD	*± 14.5*	*± 6.0*	*± 9.0*	*± 0.6*	*± 2.1*	*± 1.0*	*± 1.5*	*± 1.0*
5000 mean	/	76.7#	/	19.7	/	4.7	/	14.7
± SD	*/*	*± 11.4*	*/*	*± 1.5*	*/*	*± 0.6*	*/*	*± 1.2*
Positive control								
§ mean	616.7**	680.0**	299.3**	986.7**	143.7**	145.7**	1020.0**	165.0**
± SD	*± 58.1*	*± 17.3*	*± 23.9*	*± 220.3*	*± 4.5*	*± 10.2*	*± 166.8*	*± 47.7*

§ Information on respective positive control is reported in Material and Method section I.A.2

** Statistically significant at 1 % level (analysis of variance)

Table B.6.4.1.20-2: Mutagenicity test: Ames Salmonella Assay with Glyphosate ([redacted], 1991), second experiment

Experiment 2: Pre-incubation Test (PIT)								
Strain	**TA100**		**TA 98**		**TA 1537**		**TA 1535**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**

Cytotoxicity observed

III. CONCLUSION:

Based on the results of the present study and under the experimental conditions of the test, glyphosate was found to be non-mutagenic in the Ames test in the presence and absence of metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537) with and without metabolic activation.

The study was performed under GLP conditions and according to OECD guideline 471 (1983). When compared to the currently valid OECD guideline 471 (1997), some deviations became evident. The test was conducted with 4 valid strains only, strains like *S. typhimurium* TA 102 or *E. coli* WP2 enabling the detection of cross linking agents were not included. Further deviations to OECD guideline 471 (1997) were considered to be of minor degree and to not compromise the validity of the study. The study was considered to provide supporting information.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for gene mutation in the valid bacteria (*S. typhimurium* TA 100, TA 98, TA 1535 and TA 1537) in the presence and absence of metabolic activation under the conditions of this test. The study is considered to be supportive with restrictions due to the noted deviations.

B.6.4.1.21. *In vitro studies – bacterial gene mutation, study 21*

Data point:	CA 5.4.1/021
Report author	[redacted]
Report year	1990
Report title	Agrichem Glyphosate Active: Reverse Mutation Assay "Ames Test" Using *Salmonella Typhimurium*
Report No	300/1
Document No	Not reported
Guidelines followed in study	OECD 471 (1983), EEC Commission Directive 84/449/EEC (1984)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	An Ames test was conducted with glyphosate active (batch: 0190A, purity: not reported) in *S. typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Based on the results of a preliminary toxicity test in strain TA 100, in which no cytotoxicity was evident up to 5000 µg/plate, concentrations for the main mutagenicity test were selected. In the main mutagenicity study, two independent experiments were performed using the plate incorporation method (standard plate test).

	Triplicate cultures were exposed to test item concentrations in the range of 8 – 5000 µ/plate (first experiment) and 312.5 – 5000 µg/plate (second experiment) with and without S9 mix. Untreated, vehicle (sterile distilled water) and appropriate positive controls were included in each experiment.
Short description of results:	Precipitation of the test material in culture medium was not observed, neither in the presence nor absence of S9 mix. Cytotoxicity, evident as a reduction of the bacterial background lawn, was not observed in any experiment. However, there was a concentration-related reduction in the frequency of revertant colonies observed for most strains. In the first experiment, the frequency of revertant colonies was ≥ 50 % reduced for almost all concentrations tested (not 1000 µg/plate) for tester strain TA 1538 in the absence of metabolic activation and for tester strains ,TA 100 and TA 1535 at 5000 µg/plate in the absence of metabolic activation. In the second experiment, in the presence of S9 mix, there was a ≥ 50 % reduction in the number of revertant colonies noted for strain TA1537 at ≥ 1250 µg/plate, for strain TA 100 at ≥ 2500 µg/plate and for strain TA 1535 at 5000 µg/plate. There was no statistically significant increase in the number of revertant colonies observed in any experiment for any strain at any concentration, neither in the presence nor in the absence of metabolic activation. The number of revertant colonies induced by the vehicle control was found to be in the expected range. A strong increase in the number of revertant colonies was observed for the appropriate positive controls, demonstrating the functionality of the metabolic activation system and the validity of the test. Under the conditions of the test, glyphosate active was considered negative for gene mutation in bacteria (Ames test) in the presence and absence of metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study was considered not acceptable due to large number of deviations to current guidelines. The test was conducted in 4 valid strains only. Strains like *S. typhimurium* TA102 or *E. coli* WP2 enabling the detection of cross-linking mutagens were not included. The purity of the test material was not reported. In addition, 2-aminoanthracene was used as sole positive control substance in the presence of S9 mix and historical control data were not provided. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as agreed with GRG.

B.6.4.1.22. *In vitro studies – bacterial gene mutation, study 22*

Data point:	CA 5.4.1/022
Report author	[redacted]
Report year	1986
Report title	Report on mutagenicity tests with glyphosate (technical) of [redacted] Ames bacterial test.
Report No	Not reported
Document No	Not reported
Guidelines followed in study	No guideline followed. The study was conducted according to the main criteria of OECD 471 (1983).
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate technical (batch: not reported, purity: not reported) was evaluated for its potential to induce gene mutations in bacteria in an Ames test conducted with in *S. typhimurium* strains TA 98, TA 100,

	TA 1535 and TA 1537 and *E. coli* strain WP2 uvrA. A single experiment was performed according to the pre-incubation method. Triplicate cultures were exposed to the test item, negative and positive controls in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Three concentrations of glyphosate technical were tested, covering a concentration range of 10 – 1000 µg/plate. After at least 48 hours of incubation at 37 °C, the number of revertant colonies were counted for each plate.
Short description of results:	Evaluation of precipitation and cytotoxicity were not included in the study report. When evaluating cytotoxicity based on the number of revertant colonies in comparison to those of negative controls, treatment with glyphosate technical revealed no evidence for cytotoxicity. There was no statistically significant increase in the number of revertant colonies observed for any tester strain up to the highest tested concentration, neither in the presence nor in the absence of metabolic activation. For all conditions, the number of revertant colonies was comparable to those of negative controls. Treatment with the positive controls revealed a strong increase in the number of revertant colonies, demonstrating the functionality of the S9 mix and the sensitivity of the bacteria strains to respond to mutagenic agents. Based on the experimental results, glyphosate technical did not induce gene mutations in bacteria, neither in the presence nor in the absence of metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study was considered not acceptable due to a large number of guideline deviations. No details on the experimental performance were given and information on the test item regarding purity, batch no. and solvent were not reported. A single experiment was performed, which was not repeated in a confirmatory experiment. Further, only three concentrations were tested with an interval of factor 10 and a maximum concentration of 1000 µg/plate. There was no justification for the highest concentration given. In addition, positive controls were not included for all strains, but only for strains TA98, TA1537 and WP2uvrA. Although negative controls were included in the experiment, it was not clear whether these controls represented untreated controls or solvent controls. Further, no historical control data were provided. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation acceptable due to a large number of guideline deviations. Additional deviations that were noted: no HCD included in the study report; no correct controls.

B.6.4.1.23. *In vitro studies – bacterial gene mutation, study 23*

Data point:	CA 5.4.1/023
Report author	[redacted]
Report year	1981
Report title	Mutagenic testing of glyphosate active principle and of glyphosate product: Ames- and host mediated test
Report No	Not reported
Document No	Not reported
Guidelines followed in study	No guideline followed.
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)

Short description of study design and observations:	Glyphosate active principle (batch and purity not reported) was assessed for gene mutation in bacteria (Ames test) in *S. typhimurium* strains his-G46, TA 1537 and TA 1538. A single experiment using triplicate cultures was performed (spot test). Glyphosate was dissolved in DMSO and concentrations in the range of 1 – 1000 µg/plate were put into the central point of the agar plates. Solvent (DMSO) and positive controls (streptozotocin, 2.5 µg/plate and ICR 191, 10 µg/plate) were included. After 48 hours incubation at 37 °C, the number of revertant colonies per plate was determined.
Short description of results:	Based on the experimental findings, glyphosate active principal did not induce the frequency of revertant colonies in strains his-G46, TA 1537 and TA 1538. The positive controls streptozotocin and ICR 191 produced a marked increase in the number of revertant colonies.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study was considered not acceptable. The description of material and methods was so poor that an evaluation of the reliability of the results obtained (the test substance was considered non-mutagenic) was not possible. Further, the test was conducted in two valid strains only and only a single experiment was performed. The experimental procedure is not in line with current standard methods. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as described by the GRG.

B.6.4.1.24. *In vitro studies – bacterial gene mutation, study 24*

Data point	CA 5.4.1/024
Report author	[redacted]
Report year	1978
Report title	The report of mutagenic study with bacteria for CP 67573
Report No	ET-78-241
Document No	Not reported
Guidelines followed in study	No guideline followed, the study was conducted similarly to OECD 471 (1983)
Deviations from current test guideline OECD 471 (1997)	A single experiment using duplicate plating was performed but a justification for the missing confirmatory experiment or the missing third replicate was not provided. Instead of *E. coli* strain WP2 uvrA, strain WP2 hcr was used. 2-Aminoanthracene was used as sole positive control substance in the presence of S9 mix. Historical control data were not reported. Evaluation of cytotoxicity and precipitation were not reported, but concentrations were tested up to limit concentrations.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities. When the study was performed, GLP was not compulsory.
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered supportive due to the noted deviations.

Glyphosate (CP67573, batch: XHJ-46, purity: 98.4 %) was tested in an Ames test conducted with *S. typhimurium* strains TA 98, TA 100, TA 1535, TA1537 and TA 1538, and *E. coli* strain WP2 hcr in the presence and absence of metabolic activation (Aroclor-induced rat liver S9 fraction). A single experiment (plate-incorporation method) was performed, using test item concentrations in the range of 10 – 5000 µg/plate. Solvent (water) and appropriate positive controls were included. After incubation at 37 °C for 48 hours, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

Evaluation of precipitation and cytotoxicity were not provided in the study report. Cytotoxicity was re-evaluated by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding vehicle control. Based on these criteria, cytotoxicity was

noted at 5000 µg/plate in strains WP2 hcr, TA 100, TA 1537 and TA 98 in the absence of S9 mix and at 5000 µg/plate in strain TA 100 in the presence of S9 mix.

There was no significant increase in the number of his^+ or trp^+ revertants observed in any tester strain at any concentration when compared to solvent controls, neither in the presence nor in the absence of metabolic activation. In contrast, a strong increase in reverse mutations was observed for all positive control compounds in all tester strains, demonstrating the sensitivity of the test and the functionality of the S9 fraction.

Based on the experimental results of the present study, glyphosate is not mutagenic in the Ames plate incorporation test with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	CP67573
Description:	Not provided
Lot/Batch #:	XHJ-46
Purity:	98.4 %
Stability of test compound:	The stability of the test item at storage conditions or in solvent (vehicle) was not specified.
Solvent (vehicle) used:	Water

2. Control materials:

Negative control:	Not examined
Solvent (vehicle) control:	Water
Solvent (vehicle)/final concentration:	0.1 mL per plate.
Positive controls:	Please refer to the table below.

Strain	Metabolic activation	Mutagen	Conc. [µg/plate]
S. typhimurium strains			
TA 100	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	0.05
	+S9	2-Aminoanthracene	10.0
TA 1535	-S9	□-Propiolactone	50.0
	+S9	2-Aminoanthracene	10.0
TA 98	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	0.1
	+S9	2-Aminoanthracene	10.0
TA 1537	-S9	9-Aminoacridine	200.0
	+S9	2-Aminoanthracene	10.0
TA 1538	-S9	2-Nitrofluorene	50.0
	+S9	2-Aminoanthracene	10.0
E. coli strain			
WP2 hcr	-S9	2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	0.25
	+S9	2-Aminoanthracene	10.0

3. Metabolic activation:

S9 mix was obtained from livers of 11 weeks old male Sprague-Dawley rats with an average body weight of approx. 364 g. The animals received a single intraperitoneal injection of 500 mg/kg bw Aroclor 1254. The livers were prepared five days after treatment. Co-factor was immediately added to prepare S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
$NADP^+$	4	mM
$MgCl_2$	8	mM
S9	33	% (v/v)

4. Test organisms:

Tester strains				Bacteria batch checked for	
S. typhimurium		***E. coli***			
TA 98	✓	WP2 hcr	✓	deep rough character (rfa)	Not specified
TA 100	✓	WP2 uvrA (pKM101)		ampicillin resistance (R factor plasmid)	Not specified
TA 1535	✓			UV-light sensitivity (absence of uvrB and uvrA genes in *S. typhimurium* and *E. coli* strains, respectively)	Not specified
TA 1537	✓				
TA 1538	✓			Histidine and tryptophan auxotrophy (automatically via the spontaneous rate)	✓

5. Test concentrations:

Plate incorporation test ± S9 mix:			
Concentrations:	*10, 50, 100, 500, 1000 and 5000 µg/plate*		
Tester strains:	TA 1538, TA 1535, TA 1537, TA 98, TA 100 and WP2 hcr		
Replicates:		*Duplicates in a single experiment*	

B: STUDY DESIGN AND METHODS

1. Finalisation date: 20 Jul 1978

2. Standard plate test (plate-incorporation test, SPT):

An aliquot of 0.1 mL test solution or vehicle/positive control, an aliquot of 0.1 mL fresh bacterial culture and 0.5 mL S9 mix (in tests with metabolic activation only) were added to 2 mL of molten agar (supplemented with 0.5 mM L-histidine + 0.5 mM biotin or 0.5 mM L-tryptophan) and spread onto minimal agar plates with modified Vogel-Bonner E medium. Each concentration and the controls were tested in duplicates. After incubation at 37 °C for 48 hours, the number of bacterial colonies (his^+ or trp^+ revertants) were counted.

3. Cytotoxicity

Criteria for the evaluation of cytotoxicity have not been specified in the study report. Cytotoxicity was indicated only in case no revertant colonies were detected on a plate. Cytotoxicity was evaluated retrospectively by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding vehicle control. A cytotoxic effect was evident when the number of spontaneous revertant colonies was 50 % or less of the value in corresponding vehicle controls.

4. Statistics

Results were judged without statistical analysis.

5. Acceptance criteria

Acceptance criteria were not specified in the study report.

6. Evaluation criteria

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

Although cytotoxicity was noted in the mutagenicity study, detailed observations on cytotoxicity in individual strains were not reported. Cytotoxicity evident as no bacterial background lawn at all was observed at 5000 µg/plate in *E. coli* strain WP2 hcr in the absence of metabolic activation. Cytotoxicity was re-evaluated by comparing the presented mean numbers of revertant colonies induced by the test item to the mean number of spontaneous revertants observed in the corresponding vehicle control. A cytotoxic effect was evident when the number of spontaneous revertant colonies was 50 % or less of the value in corresponding vehicle controls. Based on these criteria, cytotoxicity was noted at 5000 µg/plate in strains WP2 hcr, TA 100, TA 1537 and TA 98 in the absence of S9 mix and at 5000 µg/plate in strain TA 100 in the presence of S9 mix.

C. SOLUBILITY

Evaluation of precipitation was not reported.

D. MUTATION ASSAY

There was no significant increase in the number of his^+ or trp^+ revertants observed in any tester strain at any concentration when compared to solvent controls, neither in the presence nor in the absence of metabolic activation.

In contrast, a strong increase in reverse mutations was observed for all positive control compounds in all tester strains, demonstrating the sensitivity of the test and the functionality of the S9 fraction.

Table B.6.4.1.24-1: The report of mutagenic study with bacteria [redacted], 1978)

Standard plate test (SPT)[X]												
Strain	**WP2 hcr**		**TA 1535**		**TA 100**		**TA 1537**		**TA 1538**		**TA 98**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**
Vehicle control												
Water mean	22.0	19.5	10.0	5.5	148.0	139.5	9.5	6.0	11.5	9.5	23.5	19.0
Test item [µg/plate]												
10 mean	21.5	43.0	3.5	2.5	145.0	122.5	5.0	3.0	20.5	13.5	27.5	21.0
50 mean	18.5	24.5	5.0	7.0	155.0	127.0	5.5	8.0	15.0	15.0	36.5	23.5
100 mean	19.0	25.0	4.5	6.0	151.5	122.0	8.0	8.5	20.5	16.0	20.0	14.5
500 mean	23.5	29.0	2.0	3.0	130.5	124.5	10.0	8.5	11.0	11.0	27.5	22.5
1000 mean	16.5	26.5	10.5	7.5	103.5	92.5	10.0	9.0	15.0	15.5	22.0	19.0
5000 mean	#	29.5	6.0	6.0	72.5[T]	43.5[T]	3[T]	4.5	6.5	13.0	6.5[T]	20.5
Positive control												
[§] mean	88.5	1972.0	355.5	336.5	>3000	1087.0	379.0	>10,000	>3000	>3000	>3000	311.0

[X] = The table shows mean values of duplicate plates which were be lately calculated based on the raw data given in study report.

Table B.6.4.1.24-1: The report of mutagenic study with bacteria ([redacted], 1978)

Standard plate test (SPT)[x]												
Strain	**WP2 hcr**		**TA 1535**		**TA 100**		**TA 1537**		**TA 1538**		**TA 98**	
Metabolic activation	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**	**- S9**	**+ S9**

§ Information on respective positive control is reported in Material and Method section I.A.2

Toxicity, no revertant colonies on the plate; T: cytotoxicity, re-evaluation as described in Material and Method section.

III. CONCLUSION:

In conclusion, based on the results of the present study and under the experimental conditions chosen, glyphosate is not mutagenic in bacteria (Ames test) with and without metabolic activation.

Assessment and conclusion by applicant:

Negative for gene mutation in bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537 and TA 1538 and *E. coli* WP2 hcr) with and without metabolic activation.

The study was performed similar to OECD guideline 471 (1997) but not GLP-compliant. It was considered supplementary, as a number of deviations to the currently valid guideline became evident. A single experiment using duplicate plating was performed but a justification for the missing confirmatory experiment or the missing third replicate was not provided. Instead of *E. coli* strain WP2 uvrA, strain WP2 hcr was used. In addition, 2-aminoanthracene was used as sole positive control substance in the presence of S9 mix and historical control data were not reported. Evaluation of cytotoxicity and precipitation were not reported, but concentrations were tested up to limit concentrations. In addition, no acceptance or evaluation criteria were specified.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for gene mutation in the valid bacteria (*S. typhimurium* TA 100, TA 98, TA 1535, TA 1537 and TA 1538 and *E. coli* WP2 hcr in the presence and absence of metabolic activation under the conditions of this test. The study is considered supportive due to the noted deviations.

B.6.4.1.25. *In vitro studies – chromosome aberration, study 1*

Data point	CA 5.4.1/025
Report author	[redacted]
Report year	1998
Report title	Glyphosate Acid: *In Vitro* Cytogenetic Assay In Human Lymphocytes
Report No	CTL/P/6050
Document No	Not reported
Guidelines followed in study	OECD 473 (1983), Commission Directive 67/548/EEC (1992), Commission Directive 92/69/EEC B.10 (1992)
Deviations from current test guideline OECD 473 (2016)	Only 200 instead of 300 cells in metaphase were evaluated per condition for test item treated cultures and only 25 metaphases were evaluated for the corresponding positive control. There are no information on numerical chromosome aberrations given (polyploidy index could not be determined). Data on the laboratory's historical control range were not provided. Positive controls were only included for the 20 hour sampling time point. A short-term exposure in the absence of metabolic activation as recommended by OECD 473 (2016) was not included. Although pH changes were observed, there was no attempt to buffer the pH change. Acceptance criteria were not specified and evaluation criteria were inconsistent with test guideline OECD 473 (2016).

Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate acid (batch: P24, purity: 95.6 %) was evaluated for its clastogenic potential *in vitro* in human lymphocytes. Cells from two different donors were treated in the presence and absence of metabolic activation (phenobarbital and β-naphthoflavone-induced rat liver S9 fraction). Duplicate cultures were exposed to test item concentrations in the range of 50 to 2000 µg/mL. Due to reductions of the pH in the culture medium at a test item concentration of 1250 µg/mL (-0.57 units) and above, the maximum concentration for the cytogenicity assay was 1250 µg/mL. Cultures treated with glyphosate acid at 100, 750 and 1250 µg/mL were selected for chromosomal aberration analysis along with the appropriate solvent (culture medium) and positive control cultures (mitomycin C without S9 mix and cyclophosphamide with S9 mix).

The cells from the first donor (male) were exposed for 20 hours in the absence of S9 mix and for 3 hours in the presence of S9 mix followed by 17 hours of incubation in test item-free medium. Sampling for chromosome preparations were made 20 hours after start of exposure in the presence and absence of S9 mix, corresponding to 68 hours after cell culture establishment. The cells from the second donor (female) were exposed for 20 and 44 hours in the absence of S9 mix, for 3 hours in the presence of S9 mix followed by 17 hours of incubation in test item-free medium and for 3 hours in the presence of metabolic activation followed by 41 hours of incubation in test item-free medium. Cells exposed for 3 hours in the presence of S9 mix were sampled 20 and 44 hours after start of exposure (68 and 92 hours after cell culture establishment). Cells exposed for 20 and 44 hours in the absence of S9 mix were sampled immediately following exposure (68 and 92 hours after cell culture establishment, respectively).

A total of 200 metaphase cells per condition were scored for structural chromosome aberrations and cytotoxicity was assessed as mitotic index (MI) and evaluated for 1000 cells per culture.

Cytotoxicity was evident at 1250 µg/mL in the absence of metabolic activation only, at the 20 hour sampling time point. In both donors, the mean mitotic activity was slightly reduced (-37 % and -33 %, respectively). There was no cytotoxicity at the 44 hour sampling time point or in the presence of metabolic activation.

Treatment with glyphosate acid did not induce a statistically or biologically significant increase in the percentage of aberrant metaphases in none of the experiments, neither in the presence nor in the absence of S9 mix. Frequencies of aberrant metaphases of solvent control cultures remained within the range of the laboratory's historical control data. The positive controls showed a clear clastogenic effect and markedly induced the number of aberrant metaphases, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Based on the results of the present study, glyphosate acid is not clastogenic to cultured human lymphocytes with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate acid

Identification:	Y04707/034
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6 %
Stability of test compound:	From information supplied by the sponsor, the test substance was stable during the period of the study.
Solvent (vehicle) used:	Culture medium

2. Control Materials:

Negative control:	The negative control corresponded to the solvent control.
Solvent (vehicle) control:	Culture medium (RPMI-1640)
Positive control:	-S9 mix: Mitomycin C (MCC): 0.2 µg/mL +S9 mix: Cyclophosphamide (CP): 50 µg/mL

3. Metabolic activation:

S9 mix was obtained from the livers of male Sprague-Dawley rats. The animals received intraperitoneal injections of phenobarbital (80 mg/kg bw) and β -naphthoflavone (100 mg/kg bw/day) for three consecutive days. The animals were sacrificed on the day following the third dose. The S9 mix was prepared on the day of culture treatment by mixing S9 fraction and co-factor solution as a 1:1 mixture.

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	150	mM
KCl	49.5	mM
NADPH-generating system		
Glucose 6-phosphate	7.5	mM
NADP Na salt	6	mM
$MgCl_2$	8	mM
S9	25	% (v/v)

4. Test organism:

Human blood samples were obtained by venepuncture in lithium heparin tubes from healthy, non-smoking donors, one male (donor 1) and one female (donor 2). Both donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.

5. Cell culture:

Medium: RPMI-1640 medium (Dutch modification) supplemented with approx. 10 % foetal bovine serum (FBS), 1.0 IU/mL heparin, 100 IU/mL penicillin and 100 µg/mL streptomycin.

Incubation: At 37 °C

Cell culture establishment prior to exposure: 0.5 mL of whole blood was added to 9.0 mL of culture medium and 5 % (v/v) phytohaemagglutinin. Cells were maintained at approximately 37 °C for 48 hours with gentle daily mixing where possible.

6. Test concentrations and number of replicates:

Exp.	Donor	Metabolic activation	Duration of exposure	Concentrations	Replicates
1	male	(- S9 mix)	20 h	50, 100*, 250, 500, 750*, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate
1	male	(+ S9 mix)	3 h[$]	50, 100*, 250, 500, 750*, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate
2	female	(- S9 mix)	20 h	50, 100*, 250, 500, 750*, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate
2	female	(+ S9 mix)	3 h[$]	50, 100*, 250, 500, 750*, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate
2	female	(- S9 mix)	44 h[#]	50, 100, 250, 500, 750, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate
2	female	(+ S9 mix)	3 h[§]	50, 100, 250, 500, 750, 1000, 1250*, 1500 and 2000 µg/mL	Duplicate

[#] The culture medium was changed after 68 h of culture initiation (= 20 hours after start of treatment)

[$] The cells were exposed for 3 hours, followed by an incubation period of 17 hours (sampling time: 20 hours after exposure

[§] The cells were exposed for 3 hours, followed by an incubation period of 41 hours (sampling time: 44 hours after exposure)

* Dose levels selected for assessment of chromosome aberration

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 03 Jul 1995 – 26 Aug 1998
Finalisation date: 29 Oct 1998

2. Preliminary cytotoxicity test:
A preliminary cytotoxicity test was not performed in this study.

3. Main cytogenicity test:

Treatment: Approximately 48 hours after cell culture establishment, 8 mL aliquots of test item formulations in medium were administered to each culture. Duplicate cultures per condition were exposed to concentrations in the range of 50 to 2000 µg/mL.
The cells from the first donor (male) were treated for 20 hours in the absence of S9 mix and for 3 hours in the presence of S9 mix, followed by 17 hours of incubation in test item-free medium. Sampling for chromosome preparations were made 20 hours after start of exposure in the presence and absence of S9 mix, corresponding to 68 hours after cell culture establishment
The cells from the second donor (female) were exposed for 20 and 44 hours in the absence of S9 mix, for 3 hours in the presence of S9 mix followed by 17 hours of incubation in test item-free medium and for 3 hours in the presence of metabolic activation followed by 41 hours of incubation in test item-free medium. Cells exposed for 3 hours in the presence of S9 mix were sampled 20 and 44 hours after start of exposure (68 and 92 hours after cell culture establishment, corresponding to 3-hour exposure followed by 17 and 41 hours of test item-free exposure). Cells exposed for 20 and 44 hours in the absence of S9 mix were sampled immediately following exposure (68 and 92 hours after cell culture establishment, respectively).
In cell cultures of the latter sampling time point (44 hours after start of exposure, 92 hours after cell culture establishment) a medium change was performed after 20 hours of exposure.
Corresponding solvent (culture medium) and positive controls (MMC without S9 mix and CP with S9 mix) were included for the 3 hours exposure period with S9 mix and for the 20 hours exposure period without S9 mix.

Spindle inhibition: Colcemid (0.4 µg/mL) was added to the cultures two hours before harvest.

Cell harvest: The cells were harvested by centrifugation and the pellets were re-suspended in 0.075 M potassium chloride solution at room temperature for approximately 10 minutes. Afterwards, the cells were centrifuged again and fixed in methanol:glacial acetic acid fixative (3:1, v/v). the fixative was removed following centrifugation and replaced with freshly prepared fixative. The process was repeated at least twice prior to slide preparation.

Slide preparation: Fixed cells were dropped on clean, moist labelled microscope slides. The slides were air-dried, stained in filtered Giemsa stain (10 % Gurr's R66) for 7 minutes, rinsed with water, air-dried again and mounted with coverslips in DPX.

Metaphase analysis The slides were coded prior to analysis and 100 cells in metaphase (200 metaphases per condition in total), where possible, were analysed from each culture for the incidence of structural chromosomal damage. Recording of chromosomal aberrations was performed according to Scott et al. (1990), including the type and frequency of observed aberrations. Chromosome gaps, breaks and minutes, multiple damage, interchanges and e.g. re-arrangements were noted. For each condition, frequencies of aberrant metaphases were calculated including and excluding gap-type aberrations.

Cytotoxicity: Mitotic indices were determined by examining 1000 lymphocytes per culture and calculating the percentage of cells in metaphase.

4. Statistics

The statistically evaluation of the percentage of metaphases showing aberrations (excluding cells with only gap-type aberrations) was performed using the (one-sided) Fisher's Exact Probability Test. Data from each treatment group in the presence and absence of S9 mix was compared to the respective solvent control value.

5. Acceptance criteria

Acceptance criteria were not specified in the study report.

6. Evaluation criteria

A test substance was judged negative if the following criteria were met:

- There was no statistically significant increase in the percentage of aberrant cells at any concentration above concurrent solvent control values.
- A statistically significant increase in the percentage of aberrant cells above concurrent solvent controls fell into the range of the laboratories historical control data.

A test substance was judged positive if there was an increase in the percentage of aberrant cells, at least at one concentration, which is substantially greater than the laboratory's historical solvent control values.

II. RESULTS AND DISCUSSION

A. ANALYTCAL DETERMINATIONS

Analytical determinations of the test substance in the solvent were not performed, as not required by the test guideline.

B. CYTOTOXICITY

Small reductions in the mean mitotic activity when compared to the respective solvent control were noted at 1250 µg/mL for the 20-hour sampling time point (68 hours after cell culture establishment) in the absence of metabolic activation only. At 1250 µg/mL, which was the highest concentration selected for chromosomal aberration analysis, in the absence of S9 mix, the mitotic indices were reduced by -37 % and -33 % for cultures of donors 1 and 2, respectively. There was no cytotoxicity observed at the 44 hour sampling time point (92 hours after cell culture establishment) in the absence of metabolic activation. In addition, there were no significant reductions in mitotic activity in all approaches in the presence of S9 mix.

Treatment with glyphosate acid caused a concentration-related reduction in the pH of the culture medium. The highest concentration of glyphosate acid selected for chromosomal aberration analysis was 1250 µg/mL, in which the pH of the culture medium was reduced by 0.57 units. Cultures treated with concentrations of glyphosate acid higher than 1250 µg/mL were therefore considered not to be suitable for chromosomal aberration analysis.

C. SOLUBILITY

Evaluation of test item precipitation was not provided in the study report. However, there was a concentration-related reduction in the pH of the culture medium in glyphosate acid treated cultures. Treatment of the culture medium with test item concentrations up to 2000 µg/mL had no significant effect on osmolality.

D. CYTOGENICITY

There was no statistically or biologically significant increase in the percentage of aberrant metaphases observed in none of the experiments, neither in the presence nor in the absence of S9 mix. Frequencies of aberrant metaphases of solvent control cultures remained within the range of the laboratory's historical control data. The positive controls mitomycin C and cyclophosphamide showed a clear clastogenic effect and markedly induced the number of aberrant metaphases, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Table B.66.4.1.25-11: Glyphosate Acid: In Vitro Cytogenetic Assay In Human Lymphocytes ([redacted] 1998), first experiment (cells from Donor 1)

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity				Judge	Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells			
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**		
Without metabolic activation; 20-hour treatment and sampling (68 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	1.00	**1.00**	0.50	**0.50**	negative	15.1
Test item	100.0	200	4.00	**3.00**	2.00	**1.50**	negative	14.70
	750.0	200	2.00	**2.00**	1.00	**1.00**	negative	12.40
	1250.0	200	2.00	**2.00**	1.00	**1.00**	negative	9.50
MMC	0.20	25	9.00	**9.00****	36.00	**36.00****	positive	7.00 ∞
With metabolic activation; 3-hour treatment, 17-hour incubation, sampling after 20 hours (68 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	4.00	**3.00**	2.00	**1.50**	negative	14.6
Test item	100.0	200	4.00	**1.00**	2.00	**0.50**	negative	13.60
	750.0	200	2.00	**0.00**	1.00	**0.00**	negative	13.80
	1250.0	200	8.00	**4.00**	4.00	**2.00**	negative	14.30
CP	50.0	25	6.00	**5.00****	24.00	**20.00****	positive	9.70 ∞

MI mitotic index: number of cells in mitosis/ number of cells, based on 1000 cells per culture
MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix
∞ Mitotic index and percentage of aberrant cells were determined from a single culture
a Mitotic index not required for selection of concentrations for chromosomal aberration analysis
** Statistically significant increase in the percentage of aberrant cells at $p < 0.01$ using the Fisher's Exact Test (one-sided)
x Total number of chromosome aberrations from 200 metaphases scored

Table B.66.4.1.25-2: Glyphosate Acid: In Vitro Cytogenetic Assay In Human Lymphocytes ([redacted] 1998), second experiment (cells from Donor 2)

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity				Judge	Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells			
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**		
Without metabolic activation; 20-hour treatment and sampling (68 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	0.00	**0.00**	0.00	**0.00**	negative	11.1
Test item	100.0	200	2.00	**2.00**	1.00	**1.00**	negative	10.40
	750.0	200	2.00	**2.00**	1.00	**1.00**	negative	9.00

Table B.66.4.1.25-2: Glyphosate Acid: In Vitro Cytogenetic Assay In Human Lymphocytes ([redacted], 1998), second experiment (cells from Donor 2)

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity				Judge	Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells			
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**		
	1250.0	201	2.00	**1.00**	1.00	**0.50**	negative	7.40
MMC	0.20	25	10.00	**9.00****	40.00	**36.00****	positive	6.70 ∞
With metabolic activation; 3-hour treatment, 17-hour incubation, sampling after 20 hours (68 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	2.00	**2.00**	1.00	**1.00**	negative	11.2
Test item	100.0	200	5.00	**5.00**	2.50	**2.50**	negative	10.70
	750.0	200	2.00	**2.00**	1.00	**1.00**	negative	10.80
	1250.0	200	3.00	**3.00**	1.50	**1.50**	negative	11.00
CP	50.0	25	8.00	**7.00***	32.00	**28.00****	positive	4.60 ∞
With metabolic activation; 3-hour treatment, 41-hour incubation, sampling after 44 hours (92 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	0.00	**0.00**	0.00	**0.00**	negative	10.1
Test item	1250.0	200	1.00	**1.00**	0.50	**0.50**	negative	9.50
Without metabolic activation; 44-hour treatment and sampling (92 hours after cell culture establishment)								
Solvent (culture medium)	800 µL/mL	200	4.00	**4.00**	2.00	**2.00**	negative	11.2
Test item	1250.0	200	3.00	**3.00**	1.50	**1.50**	negative	11.30

MI mitotic index: number of cells in mitosis/ number of cells, based on 1000 cells per culture
MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix
∞ Mitotic index and percentage of aberrant cells were determined from a single culture
a Mitotic index not required for selection of concentrations for chromosomal aberration analysis
** Statistically significant increase in the percentage of aberrant cells at $p < 0.01$ using the Fisher's Exact Test (one-sided)
x Total number of chromosome aberrations from 200 metaphases scored

III. CONCLUSION:

In conclusion, glyphosate acid was not clastogenic to human lymphocytes *in vitro*, either in the presence or absence of metabolic activation.

Assessment and conclusion by applicant:
Negative for cytogenicity in peripheral human lymphocytes with and without metabolic activation.
The study was performed under GLP conditions and in accordance with OECD guideline 473 (1983). When compared to the currently valid guideline OECD 473 (2016), a number of deviations were noted. Only 200 metaphase cells were investigated and numerical chromosome aberrations were obviously not included in the evaluation. A short-term exposure in the absence of S9 mix was not included in the experiment and positive controls were only included for the 20-hour sampling time point.

Further deviations were considered to be of minor degree and to not compromise the outcome of the study. The study is considered to provide supporting information

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for cytogenicity in peripheral human lymphocytes in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.26. *In vitro studies – chromosome aberration, study 2*

Data point	CA 5.4.1/026
Report author	[redacted]
Report year	1996
Report title	Technical glyphosate: Chromosome aberration test in CHL cells *in vitro*
Report No	434/015
Document No	Not reported
Guidelines followed in study	Not specified, experimental procedure similar to OECD 473 (2016)
Deviations from current test guideline OECD 473 (2016)	Only 200 cells in metaphase were evaluated, whereas the evaluation of 300 metaphases is recommended according to OECD guideline 473 (2016). No historical control data from the testing laboratory provided, but values were compared to published control values. Although pH changes were observed and the maximum concentration was limited based on pH changes, there was no attempt to buffer the pH change. Cytotoxicity was measured by counting the number of cells at the end of the culture period relative to control, whereas OECD 473 (2016) recommends to measure the relative population doubling or the relative increase in cell count. Acceptance and evaluation criteria were inconsistent with test guideline OECD 473 (2016). The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: H95D161A, purity: 95.3 %) was tested for clastogenic effects *in vitro* in Chinese hamster lung (CHL) cells in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Test item concentrations were selected based on the results of a preliminary cytotoxicity test. Due to reductions of the pH in culture medium at test item concentrations > 1250 µg/mL, the maximum concentration for the main mutagenicity assay was 1250 µg/mL. Duplicate cultures were exposed to the test substance at concentrations of 39, 78.1, 156.25, 312.5, 625 and 1250 µg/mL for 24 and 48 hours in the absence of metabolic activation and for 6 hours in the presence and absence of metabolic activation. Concurrent vehicle (culture medium) and positive controls (mitomycin C without S9 mix and cyclophosphamide with S9 mix) were included for experiments with and without metabolic activation, respectively.

The cells were sampled after 24 and 48 hours of exposure without S9 mix and 24 h after start of exposure for the 6 hour exposure with and without S9 mix. A total of 200 metaphases per condition were scored for structural and numerical chromosome aberrations. Cytotoxicity was assessed as percentage of growth inhibition when compared to solvent controls.

There was no cytotoxicity observed at any concentration of any sampling time point, neither in the presence, nor in the absence of metabolic activation.

There was no statistically significant increase in the frequency of cells with chromosomal aberrations at any dose level in any treatment group. In addition, the test material did not induce a significant increase in the numbers of polyploid metaphase cells in any of the tested conditions with or without S9 mix. Frequencies of aberrant metaphases of vehicle control cultures were within the expected range. The positive controls gave highly significant increases in the frequency of aberrant metaphase cells, indicating that the metabolic activation system was satisfactory and that the test method itself was operating as expected.

Based on the results of the present study, there is no evidence for induction of chromosome aberrations by glyphosate technical with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Technical Glyphosate

Identification:	Not specified
Description:	White powder
Lot/Batch #:	H95D161A
Purity:	95.3 %
Stability of test compound:	The stability of the test item at storage conditions (room temperature) was guaranteed for > 2 years. The stability of the test item in the solvent (vehicle) was at the responsibility of the sponsor and not further specified.
Solvent (vehicle) used:	Culture medium (Eagle's Minimal Essential medium with Earle's Salts)

2. Control Materials:

Negative control:	The negative control corresponded to the solvent control.
Solvent (vehicle) control:	Culture medium (Eagle's Minimal Essential medium with Earle's Salts)
Positive control:	-S9 mix: Mitomycin C (MCC): 0.05 µg/mL +S9 mix: Cyclophosphamide (CP): 10 µg/mL

3. Metabolic activation:

S9 mix was obtained from the livers of male Sprague-Dawley rats, weighing approx. 200 g. The animals received a single intraperitoneal injection of Aroclor 1254. Five days after administration, the S9 fraction was prepared. Prior to the experiment, an aliquot of S9 mix fraction was mixed with standard co-factors.

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.4)	30	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	5	% (v/v)

4. Test organism:

CHL cells were used, established from the lung of Chinese hamster. The cells have an average generation time of approximately 11 hours. It was not reported whether the cells were screened for mycoplasma contamination.

5. Cell culture:

Medium:	Eagle's MEM medium supplemented with 10 % foetal calf serum and antibiotics
Incubation:	At 37 °C with 5 % CO_2 in air
Cell culture establishment prior to exposure:	The cells were seeded approx. 48 hours prior to treatment in 25 cm^2 flasks. 0.15 x 10^6 cells were seeded per flask for the 6 and 24 h cultures and 0.075 x 10^6 cells were seeded per flask for the 48 h cultures.

6. Test concentrations and number of replicates:

(a) Preliminary cytotoxicity assay:

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	24 h	19.5, 39.1, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/mL	Duplicate
-S9 mix	48 h	19.5, 39.1, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/mL	Duplicate
± S9 mix	6 h	19.5, 39.1, 78.13, 156.25, 312.5, 625, 1250, 2500 and 5000 µg/mL	Duplicate

(b) Main cytogenicity test:

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	24 h	39, 78.1, 156.25, 312.5*, 625* and 1250* µg/mL	Duplicate
-S9 mix	48 h	39, 78.1, 156.25, 312.5*, 625* and 1250* µg/mL	Duplicate
± S9 mix	6 h	39, 78.1, 156.25, 312.5*, 625* and 1250 µg/mL	Duplicate

* Dose levels selected for assessment of chromosome aberration

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 30 Aug 1995 – 04 Jan 1996
Finalisation date: 18 Jan 1996

2. Preliminary cytotoxicity test:
In a preliminary test, CHL cells were treated at concentrations in the range of 19.5 to 5000 µg/mL each with and without metabolic activation. Duplicate cell cultures were exposed to the test item or solvent. The cells were exposed for 24 and 48 hours without metabolic activation and for 6 hours with and without metabolic activation, followed by an 18-hour recovery period in treatment-free medium. Growth inhibition was estimated by counting the number of cells at the end of the culture period and expressing the cell count as a percentage of the concurrent vehicle control value. In addition, slides were prepared from the cells in order to check for the presence of cells in metaphase.

3. Main cytogenicity test:

Treatment: After the 48 hour pre-incubation period, treatment for the main mutagenicity test was initiated. Based on the results of the preliminary cytotoxicity test, duplicate cultures were exposed to test item concentrations in the range of 39 to 1250 µg/mL for 24 and 48 hours in the absence of metabolic activation and for 6 hours in the presence and absence of metabolic activation. Corresponding solvent and positive controls (MMC without S9 mix and CP with S9 mix) were included.
In the absence of S9 mix, chromosome preparations were made at 24 and 48 hours after start of treatment. In case of 6 hour exposure in the presence and absence of S9 mix, the cells were incubated for 18 hours following exposure and chromosome preparations were made at 24 h after start of exposure.

Spindle inhibition: Colcemid (0.1 µg/mL) was added to the cultures two hours before harvest.

Cell harvest: The cells were harvested by centrifugation of trypsinised cultures and re-suspended in hypotonic potassium chloride solution (0.075 M) for 10 minutes. Afterwards, the cells were centrifuged again and fixed in methanol:glacial acetic acid fixative (3:1, v/v). The fixative was changed several times and the cells stored at 4 °C for sufficient time to ensure complete fixation.

Slide preparation: Fixed cells were dropped onto clean, wet microscope slides and left to air dry. Each slide was labelled with the appropriate identification data. Air-dried slides were stained in 2 % Gurrs Giemsa R66 for 5 minutes, rinsed, dried and coverslipped in mounting medium.

Metaphase analysis Where possible, 100 consecutive well-spread metaphases were examined per culture (200 metaphase cells per condition in total), using microscopic assessment.

Concentrations in the range of 312.5 to 1250 µg/mL were evaluated for the number of chromosomes, chromosome gaps, breaks or re-arrangements. Cells with 28-31 chromosomes were scored as aneuploidy cells. In addition, the % incidence of polyploid cells (defined as metaphase cells with > 31 chromosomes) was noted. The percentage of cells showing structural chromosome aberrations (gaps, breaks and exchanges) were calculated including and excluding gap-type aberrations.

Cytotoxicity: Growth inhibition was estimated by counting the number of cells at the end of the culture period and expressing the cell count as a percentage of the concurrent vehicle control value.

4. Statistics

Statistical significance on the number of aberrant metaphases and polyploid cells when compared with those of corresponding solvent controls was identified using the Fisher's Exact test.

5. Acceptance criteria

The cytogenicity test was considered acceptable if the percentage of cells with chromosomal aberrations was within the range of aberration frequencies acceptable for control cultures, which are commonly in the range of 0 – 3 % (Ishidate (1987))[3].

6. Evaluation criteria

A positive response was recorded for a particular treatment if the percentage of cells with aberrations (gaps included) was ≥ 10 %. For polyploid cells, an incidence > 10 % is generally considered as positive.
An equivocal response was recorded for values between 5 and 10 %.
A negative response was obtained if the percentage of cells with aberrations was < 5 %.

II. RESULTS AND DISCUSSION

A. ANALYTCAL DETERMINATIONS

Analytical determinations of the test substance in the solvent were not determined as not required by the test guideline.

B. CYTOTOXICITY

Preliminary toxicity test:

In the preliminary cytotoxicity test cytotoxicity was observed at ≥ 2500 µg/mL after 24 hours of exposure and at ≥ 1250 µg/mL after 48 hours of exposure, both in the absence of metabolic activation. After 6 hours of exposure in the absence of S9 mix, cytotoxicity was evident at 5000 µg/mL. Growth inhibition was also observed at lower doses in the range of 19.5 – 1250 µg/mL, however, there was no dose-response relationship and no cytotoxicity at 2500 µg/mL. There was no cytotoxicity after 6 hours of exposure in the presence of S9 mix.
After 6 hours of exposure, metaphase cells were observed in the presence and absence of S9 mix up to a concentration of 5000 µg/mL. After 24 and 48 hours of exposure in the absence of metabolic activation, metaphases were present at concentrations up to 2500 µg/mL.
In addition, there was a dose-related reduction in pH value. At 2500 and 5000 µg/mL, the pH was reduced by ≥ 1 unit.
Based on the observations in pH changes in the preliminary test, the maximum dose level selected for the main study was 1250 µg/mL.

Main mutagenicity test

In the main mutagenicity test there was no cytotoxicity observed up the highest tested concentration, both in the presence and absence of metabolic activation.

C. SOLUBILITY

Evaluation of test item precipitation was not provided in the study report. However, there was a dose-related decrease in the pH value noted in all experiments. At 2500 and 5000 µg/mL, the pH was reduced by ≥ 1 unit.

D. CYTOGENICITY

[3] Ishidate (1987): Data Book of Chromosomal Aberration Test *in Vitro*

There was no statistically significant increase in the frequency of cells with chromosomal aberrations at any dose level in any treatment group. In addition, the test material did not induce a significant increase in the numbers of polyploid metaphase cells in any of the tested conditions with or without S9 mix. Frequencies of aberrant metaphases of vehicle control cultures were within the expected range. The positive controls mitomycin C in the absence of S9 mix and cyclophosphamide in the presence of S9 mix gave highly significant increases in the frequency of aberrant metaphase cells. No statistically significant increase was observed for cyclophosphamide after 6 hours of exposure in the absence of S9 mix. The test results indicate that the metabolic activation system was functioning satisfactory and that the test method itself was operating as expected.

Table B.64.1.26-1*:* Technical glyphosate: Chromosome aberration test in CHL cells in vitro ([redacted] 1996), 24 and 48 h incubation without metabolic activation

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Growth inhibition [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		
Without metabolic activation; 24-hour treatment and sampling									
Solvent (culture medium)		200	3.00	**3.00**	1.50	**1.50**	1.00	negative	100.0
HCD[#] range					0-3				
Test item	312.5	200	2.00	**0.00**	1.00	**0.00**	1.00	negative	94.00
	625.0	200	1.00	**1.00**	0.50	**0.50**	0.00	negative	115.00
	1250.0	200	2.00	**1.00**	1.00	**0.50**	0.00	negative	108.00
MMC	0.05	200	37***	**32*** **	18.50	**16.00**	1.00	positive	97.00
Without metabolic activation; 48-hour treatment and sampling									
Solvent (culture medium)		200	3.00	**2.00**	1.50	**1.00**	1.00	negative	100.0
HCD[#] range					0-3				
Test item	312.5	200	7.00	**5.00**	3.50	**2.50**	3.00	negative	110.00
	625.0	200	6.00	**5.00**	3.00	**2.50**	3.00	negative	107.00
	1250.0	200	6.00	**4.00**	3.00	**2.00**	1.00	negative	100.00
MMC	0.05	200	61***	**56*** **	40.70	**37.30**	0.00	positive	84.00

[#] HCD Historical control data from published literature cited in the study report

MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix

[x] Total number of chromosome aberrations from 200 metaphases scored

*** $p < 0.001$ with Fisher's Exact Test

Table B.6.4.1.26-2: **Technical glyphosate: Chromosome aberration test in CHL cells in vitro ([redacted] 1996), 6 h incubation with and without metabolic activation**

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Growth inhibition [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		
With metabolic activation; 6-hour treatment, 18-hour incubation, sampling after 24-hours									
Solvent (culture medium)		200	6.00	**4.00**	3.00	**2.00**	1.00	negative	100.0
HCD[#] range					0-3				
Test item	312.5	200	1.00	**1.00**	0.50	**0.50**	0.00	negative	97.00
	625.0	200	5.00	**2.00**	2.50	**1.00**	0.00	negative	95.00
	1250.0	200	5.00	**3.00**	2.50	**1.50**	0.00	negative	103.00
CP	10	200	57***	**48*** **	38.00	**32.00**	0.00	positive	66.00

Table B.6.4.1.26-2: **Technical glyphosate: Chromosome aberration test in CHL cells in vitro ([redacted] 1996), 6 h incubation with and without metabolic activation**

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Growth inhibition [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		
Without metabolic activation; 6-hour treatment, 18-hour incubation, sampling after 24-hours									
Solvent (culture medium)		200	1.00	**1.00**	0.50	**0.50**	3.00	negative	100.0
HCD[#] range					0-3				
Test item	312.5	200	2.00	**2.00**	1.00	**1.00**	0.00	negative	103.00
	625.0	200	2.00	**2.00**	1.00	**1.00**	0.00	negative	101.00
	1250.0	200	2.00	**1.00**	1.00	**0.50**	2.00	negative	114.00
CP	10	200	3.00	**3.00**	1.50	**1.50**	0.00	negative	113.00

[#] HCD Historical control data from published literature cited in the study report

MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix

[x] Total number of chromosome aberrations from 200 metaphases scored

*** $p < 0.001$ with Fisher's Exact Test

III. CONCLUSION:

In conclusions, Technical Glyphosate did not induce any statistically significant, dose-related increase in the frequency of cells with chromosome aberrations or in the frequency of polyploid cells, neither in the presence nor absence of a liver enzyme metabolising system or after various exposure times. Technical Glyphosate is therefore considered to be non-c1astogenic to CHL cells in vitro.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Negative for cytogenicity in Chinese hamster lung cells with and without metabolic activation.

The study was performed under GLP conditions and the experimental procedure was similar to OECD guideline 473 (2016), except for some minor deviations. Only 200 metaphase cells were investigated, which was the number of metaphases to be analysed according to previous OECD guidelines 473. In addition, cytotoxicity was evaluated by counting the number of cells at the end of the culture period relative to control, whereas the currently valid guideline (2016) recommends the evaluation based on relative population doubling or relative increase of the cell count. These and further deviations were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for cytogenicity in Chinese hamster lung cells in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.27. *In vitro studies – chromosome aberration, study 3*

Data point	CA 5.4.1/027
Report author	[redacted]
Report year	1995

Report title	HR-001: *In vitro* cytogenicity test
Report No	IET 94-0143
Document No	Not reported
Guidelines followed in study	OECD 473 (1983), U.S. EPA FIFRA Guidelines, Subdivision F (1991) and Japanese MAFF (1985)
Deviations from current test guideline OECD 473 (2016)	Only 200 cells in metaphase were evaluated, whereas the evaluation of 300 metaphases is recommended according to OECD guideline 473 (2016). Historical control data was provided for untreated and solvent control cultures only, but not for the positive control substances. pH measurements were not performed. Cytotoxicity was evaluated based on mitotic indices, whereas OECD 473 (2016) recommends to measure the relative population doubling or the relative increase in cell count. Acceptance and evaluation criteria were inconsistent with those specified in test guideline OECD 473 (2016). The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (HR-001, batch: 940908-1, purity: 95.68 %) was tested *in vitro* for its potential to induce chromosomal aberrations in Chinese hamster lung (CHL) cells in the presence and absence of metabolic activation (phenobarbital and 5,6 benzoflavone-induced rat liver S9 fraction). Dose levels were chosen based on the results of a preliminary cytotoxicity test, in which cytotoxicity was observed after 48 hours of treatment at 1000 µg/mL in the absence of S9 mix and after 6 hours of treatment at 2000 µg/mL in the presence of S9 mix.

Duplicate cultures were exposed to the test substance at concentrations of 125, 250, 500 and 1000 µg/mL for 24 hours without S9 mix, at concentrations of 62.5, 125, 250 and 500 µg/mL for 48 hours without S9 mix and at concentrations of 250, 500, 1000 and 2000 µg/mL for 6 hours in the presence and absence of S9 mix. Untreated cultures, solvent controls (Hanks´ balanced salt solution) and positive controls (mitomycin C without S9 mix and benzo(a)pyrene with S9 mix) were included for experiments with and without metabolic activation, respectively.

The cells were sampled after 24 and 48 hours of exposure without S9 mix and 24 h after start of exposure for the 6 hour exposure with and without S9 mix. A total of 200 metaphases per condition were scored for structural and numerical chromosome aberrations. Cytotoxicity was assessed as mitotic index (MI) and evaluated for 1000 cells per culture.

Cytotoxicity was observed after 48 hours of treatment at 1000 µg/mL in the absence of S9 mix and after 6 hours of treatment at 2000 µg/mL in the presence of S9 mix. Due to the high cytotoxicity, no cells for chromosome preparations were obtained at these concentrations.

Treatment with the test item did not induce a significant increase in the number of aberrant metaphases in any of the tested conditions. After 24 and 48 hours of treatment in the absence of metabolic activation and after 6 hours exposure in the presence and absence of metabolic activation, the number of chromosome aberrations with and without gaps was comparable to those of controls. In addition, there was no increase in the frequencies of polyploid metaphases in any of the tested conditions with or without S9 mix. Frequencies of aberrant metaphases of untreated and solvent control cultures remained within the range of the laboratory's historical control data. The positive controls showed a clear clastogenic effect and markedly increased the number of aberrant metaphases when compared to untreated and solvent control cultures, demonstrating the validity of the test system.

Based on the results of the present study, there is no evidence for a clastogenic potential of glyphosate technical with and without metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. **Test material:** Glyphosate technical
   Identification: HR-001
   Description: White crystals
   Lot/Batch #: 940908-1
   Purity: 95.68 %
   Stability of test compound: The stability of the test item at storage conditions (at room temperature) or in the solvent (vehicle) were not specified.
   Solvent (vehicle) used: Hank's balances salt solution (HBSS) and culture medium

2. **Control Materials:**
   Negative control: Untreated cell cultures were included.
   Solvent (vehicle) control: Hank's balance salt solution (10 % final concentration in medium)
   Positive control: -S9 mix: Mitomycin C (MCC): 0.1 µg/mL in physiological saline
   +S9 mix: Benzo(a)pyrene (B(a)P): 40 µg/mL in DMSO

3. **Metabolic activation:**

S9 mix was purchased from [redacted]). The homogenate was obtained from the livers of 7 weeks old male Sprague-Dawley rats, weighing 192 – 229 g. The animals received intraperitoneal injections of phenobarbital (30 mg/kg bw on Day 1, each 60 mg/kg bw on Days 2, 3 and 4) and 5,6 benzoflavone on Day 3. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factor.

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADH	4	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	30	% (v/v)

4. **Test organism:**

CHL cells were used, established from the lung of Chinese hamster. Stocks of passage number 11 were stored in liquid nitrogen and thawed immediately before use. Each batch used for mutagenicity testing was screened for mycoplasma contamination.

5. **Cell culture:**
   Medium: Eagle's MEM medium supplemented with 10 % newborn calf serum
   Incubation: At 37 °C in a humidified atmosphere of 5 % CO_2

Cell culture establishment prior to exposure
Preliminary cytotoxicity assay: CHL cells were seeded at density of 1.0 x 10^5 cells in 5 mL of medium and incubated for 48 h
Main cytogenicity test: CHL cells were seeded at density of 2.0 x 10^5 cells in 10 mL of medium and incubated for 48 h

6. Test concentrations and number of replicates:

(a) Preliminary cytotoxicity assay (growth inhibition test):

Metabolic activation	**Duration of exposure**	**Concentrations**	**Replicates**
-S9 mix	24 h	3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/mL	Duplicate
-S9 mix	48 h	3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/mL	Duplicate
+ S9 mix	6 h	3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 µg/mL	Duplicate
+ S9 mix	6 h*	1000, 2000, 3000, 4000 and 5000 µg/mL	Duplicate

* Repeated growth inhibition test with metabolic activation at higher concentrations

(b) Main cytogenicity test:

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	24 h	125, 250, 500 and 1000 µg/mL	Duplicate
-S9 mix	48 h	62.5, 125, 250 and 500 µg/mL	Duplicate
± S9 mix	6 h	250, 500, 1000 and 2000 µg/mL	Duplicate

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 13 Mar – 09 May 1995
Finalisation date: 29 May 1995

2. Preliminary cytotoxicity test:
In a preliminary test, CHL cells were seeded at density of 1.0 x 10^5 cells in 5 mL of medium and incubated for 48 h to establish the cell culture. The cells were treated at concentrations in the range of 3.9 to 1000 µg/mL in the presence and absence of metabolic activation. Without S9 mix (direct method) duplicate cultures each were exposed for 24 and 48 hours and relative cell growth was measured by comparing with the staining density in the concurrent solvent control. In the presence of S9 mix, duplicate cell cultures were exposed for 6 hours, followed by an incubation period of 18 hours. About 24 hours after start of exposure, the relative cell growth was determined. As no reduction of cell growth was observed in the presence of metabolic activation, a second growth inhibition test was performed using test item concentrations in the range of 1000 – 5000 µg/mL in the presence of S9 mix.

3. Main cytogenicity test:

Treatment: CHL cells were seeded at a density of 2.0 x 10^5 cells with 10 mL of medium and incubated for 48 h to establish the cell culture. Based on the results of the preliminary cytotoxicity test, the duplicate cultures were exposed to test item concentrations of 125 – 1000 µg/mL for 24 hours in the absence of metabolic activation, at concentrations of 62.5 – 500 µg/mL for 48 h in the absence of metabolic activation and at concentrations of 250 – 2000 µg/mL for 6 hours in the presence and absence of metabolic activation. Corresponding untreated negative control, solvent and positive controls (MMC without S9 mix for 24 and 48 hour treatment and B(a)P with S9 mix) were included.
In the absence of S9 mix, chromosome preparations were made at 24 and 48 hours after start of treatment. In case of 6 hour exposure, the cells were incubated for 18 hours following exposure and chromosome preparations were made at 24 h after start of exposure. The 6-hour experiments in the presence and absence of S9 mix were set-up as parallel experiments with the one without S9 being a concurrent control experiment against S9 mix treatment (all conditions the same as the exept for the adding S9 mix; in both settings, B(a)P was used as positive control).

Spindle inhibition: Colchicine (0.5 µg/mL) was added to the cultures two hours before harvest.

Cell harvest: The cells were detached by 0.25 % trypsin and swollen with a hypotonic potassium chloride solution (0.075 mM) for 15 minutes at room temperature. Afterwards the cells were fixed with Carnoy's solution (methanol:acetic acid = 3:1) and dropped on glass slides.

Slide preparation: Fixed cells were dropped on glass slides and air-dried. Two slides were prepared per culture and labelled with code numbers. Air-dried slides were stained with 2 % Giemsa solution for 15 minutes at room temperature.

Metaphase analysis A total number of 200 metaphase cells per condition (100 metaphase cells per culture) were examined by light microscopy. Diploid metaphase cells which possessed the typical karyotype of CHL cells and polyploid metaphase cells were analysed for chromosomal aberrations. The following data were recorded:

- Number and frequency of polyploid cells
- Number and frequency of each structural chromosome aberrations
- Number and frequency of metaphase cells with structural chromosome aberrations. Chromosome gaps, -breaks, -exchanges and chromatid breaks and -exchanges, fragmentation and other structural chromosome aberrations such as multiple aberration were recorded.
- Numerical chromosome aberrations. Only polyploid cell having 3 or more copies of haploid number of chromosomes was scored as a numerical chromosome aberration cell.

Cytotoxicity: Mitotic indices were calculated based on the number of cells in metaphase observed per 1000 cells scored.

4. Statistics

The number of aberrant metaphases and polyploid cells at each dose were statistically compared with those of corresponding solvent controls using a chi-square test

5. Acceptance criteria

The cytogenicity test was considered valid if the following criteria were met:

- The frequencies of the aberrant metaphases in the solvent control groups were within the range of the laboratory's historical negative control range (mean ± 3x SD).
- The frequency of the aberrant metaphases in the positive control groups were 10 % or more.

6. Evaluation criteria

A test substance was judged negative if there was no significant increase in the frequencies of aberrant metaphases or polyploid cells at any dose. A test substance was judged positive if reproducible and significant increases in the frequencies of aberrant metaphases or polyploid cells were observed with a dose-related response. Both biological and statistical significance were considered together in a final evaluation.

II. RESULTS AND DISCUSSION

A. ANALYTCAL DETERMINATIONS

Analytical determinations of the test substance in the solvent were not performed, as not required by the test guideline.

B. CYTOTOXICITY

Preliminary cytotoxicity test

In the preliminary cytotoxicity test, cytotoxicity was observed in the presence and absence of metabolic activation. Without S9 mix (24 and 48 h treatment), a reduction in relative cell growth by 50 % or more were observed at 500 and 1000 µg/mL, respectively. With S9 mix, no cell growth inhibition effect was observed in the first growth inhibition test up to 1000 µg/mL. A second growth inhibition test with S9 mix was performed using concentrations up to 5000 µg/mL. Cell growth inhibition of over 50 % was observed at 2000 µg/mL and above. No reduction in relative cell growth by 50 % or more was observed at any concentration in cells exposed for 6 hours without S9 mix.

It was noticed that in both experiments the color of the culture medium was turned to yellow at 500 µg/mL or more, indicating a decline of pH.

Results obtained in this preliminary cytotoxicity test were used to select concentrations for the main cytogenetic assay. The highest test compound concentration was expected to reduce mitotic index to approximately 50 % compared to corresponding solvent controls. Based on the findings of the preliminary test, the highest concentrations for the main cytogenicity assay were chosen to be 1000 µg/mL in the absence of S9 mix and 2000 µg/mL in the presence of S9 mix.

Table B.64.1-27-1: Preliminary growth inhibition test

Concentration (µg/mL)	Relative cell growth (%)			
	-S9 mix 24 h	-S9 mix 48 h	+S9 mix 6-18 h (1st exp.)	+S9 mix 6-18 h (2nd exp.)
Solvent control (HBSS)	100	100	100	100
3.9	100	100	100	-
7.8	97	101	99	-
15.6	100	101	108	-
31.3	96	110	104	-
62.5	100	106	97	-
125	102	99	103	-
250	92	82	106	-
500	74	46	112	-
1000	22	9	106	100
2000	-	-	-	22
3000	-	-	-	38
4000	-	-	-	27
5000	-	-	-	25

Main mutagenicity test

In the main mutagenicity test, excessive cytotoxicity was observed after 48 hour treatment at 1000 µg/mL in the absence of S9 mix and after 6 hours treatment at 2000 µg/mL in the presence of S9 mix. Due to the high cytotoxicity, no cells for chromosome preparations were obtained at these concentrations.

It was noticed that in both treatments (with and without S9 mix) pH of the culture medium of the cultures treated at 500, 1000 and 2000 µg/mL went down.

C. SOLUBILITY

Precipitation of the test item was not reported up to the highest tested concentrations, neither in the presence, nor in the absence of metabolic activation. However, a decrease in pH was noted in all experiments at ≥ 500 µg/mL with and without S9 mix, indicated by a medium color change to yellow. As there was no significant increase in the number of aberrant metaphases at any concentration in any test, this decrease in pH does not have any impact.

D. CYTOGENICITY

There was no significant increase in the number of aberrant metaphases observed in none of the experiments, neither in the presence nor in the absence of S9 mix. After 24 and 48 hours of treatment in the absence of metabolic activation and after 6 hours exposure in the presence and absence of metabolic activation, the number of chromosome aberrations with and without gaps was comparable to those of controls. In addition, there was no increase in the frequencies of polyploid metaphases in any of the tested conditions with or without S9 mix. Frequencies of aberrant metaphases of untreated and solvent control cultures remained within the range of the laboratory's historical control data. The positive controls mitomycin C (- S9) and benzo(a)pyrene (+ S9) showed a clear clastogenic effect and markedly increased the number of aberrant metaphases when compared to untreated and solvent controls, demonstrating the sensitivity of the test and the functionality of the S9 mix. In the 6-hour treatment without S9 (concurrent control experiment against S9 mix treatment), benzo(a)pyrene showed no significant increase in chromosome aberrations (as expected), as this requires metabolic activation.

Table B.6.4.1.27-2: *In vitro* cytogenicity test ([redacted] 1995), 24 and 48 h incubation without metabolic activation

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI (%)	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		
Without metabolic activation; 24-hour treatment and sampling									
Untreated	0.0 %	200	3.00	**1.00**	1.50	**0.50**	0.00	negative	6.1
HCD[#] mean	0.0 %				1.12	**0.50**	0.37		
± SD					± 0.93	**± 0.59**	± 0.56		
Solvent (HBSS)	10.0 %	200	0.00	**0.00**			1.00	negative	6.0
HCD[#] (DMSO) mean	0.5 %				1.32	**0.61**	0.43		
± SD					± 0.75	**± 0.55**	± 0.52		
Test item	125.0	200	1.00	**1.00**	0.50	**0.50**	1.00	negative	6.70
	250.0	200	3.00	**1.00**	1.50	**0.50**	1.00	negative	5.90
	500.0[b]	200	4.00	**1.00**	2.00	**0.50**	0.00	negative	5.60
	1000.0[bc]	200							
MMC	0.1	200	101.00	**98.00**	50.50	**49.00**	1.00	positive	3.30
Without metabolic activation; 48-hour treatment and sampling									
Untreated	0.0 %	200	3.00	**2.00**	1.50	**1.00**	1.00	negative	2.5
HCD[#] mean	0.0 %				1.18	**0.50**	0.48		
± SD					± 1.22	**± 0.66**	± 0.51		
Solvent (HBSS)	10.0 %	200	0.00	**0.00**	0.00	**0.00**	1.00	negative	3.4
HCD[#] (DMSO) mean	0.5 %				1.18	**0.65**	0.47		
± SD					± 0.86	**± 0.61**	± 0.49		
Test item	62.5	200	3.00	**0.00**	1.50	**0.00**	0.00	negative	3.00
	125.0	200	2.00	**1.00**	1.00	**0.50**	0.00	negative	2.90
	250.0	200	5.00	**2.00**	2.50	**1.00**	2.00	negative	3.10
	500.0[b]	200	5.00	**2.00**	2.50	**1.00**	0.00	negative	3.00
MMC	0.1	200	144.00	**141.00**	72.00	**70.50**	2.00	positive	2.60

[#] HCD Historical control data based on 39 cytogenicity tests performed in the laboratory from December 1988 to December 1994

MI mitotic index: number of cells in mitosis/ number of cells, based on 1000 cells per culture
PI: polyploid index: (number of polyploid + endoreduplicated cells)/number of cells in mitosis, based on 200 metaphases

MMC Mitomycin C, positive control without S9 mix; B(a)P: Benzo(a)pyrene, positive control with S9 mix

[b] The color of the culture medium turned yellow after addition of the test substance

[c] No chromosome preparations due to cytotoxicity

Table B.6.4.1.27-2: *In vitro* cytogenicity test ([redacted], 1995), 24 and 48 h incubation without metabolic activation

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI (%)	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		

[x] Total number of chromosome aberrations from 200 metaphases scored

Table B.6.4.1.27-3: *In vitro* cytogenicity test ([redacted]1995), 6 h incubation with and without metabolic activation

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI (%)	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		
With metabolic activation; 6-hour treatment, 18-hour incubation, sampling after 24-hours									
Untreated	0.0 %	200	4.00	**1.00**	2.00	**0.50**	0.00	negative	4.9
HCD[#] mean	0.0 %				1.38	**0.52**	0.48		
± SD					± 1.22	**± 0.69**	± 0.70		
Solvent (HBSS)	10.0 %	200	3.00	**1.00**	1.50	**0.50**	0.00	negative	6.3
HCD[#] (DMSO) mean	0.5 %				1.41	**0.72**	0.43		
± SD					± 0.98	**± 0.67**	± 0.38		
Test item	250.0	200	2.00	**2.00**	1.00	**1.00**	0.00	negative	6.70
	500.0[b]	200	4.00	**2.00**	2.00	**1.00**	0.00	negative	5.60
	100.00[b]	200	3.00	**1.00**	1.50	**0.50**	1.00	negative	7.20
	2000.0[bc]	200							
B(a)P	40.0	200	79.00	**77.00**	39.50	**38.50**	0.00	positive	3.80
Without metabolic activation; 6-hour treatment, 18-hour incubation, sampling after 24-hours									
Untreated	0.0 %	200	4.00	**3.00**	2.00	**1.50**	0.00	negative	5.3
Solvent (HBSS)	10.0 %	200	2.00	**0.00**	1.00	**0.00**	2.00	negative	5.7
Test item	250.0	200	4.00	**2.00**	2.00	**1.00**	0.00	negative	5.10
	500.0	200	2.00	**1.00**	1.00	**0.50**	0.00	negative	4.90
	1000.0[b]	200	2.00	**1.00**	1.00	**0.50**	1.00	negative	5.70
	2000.0[bc]	200							
B(a)P	40.0	200	1.00	**1.00**	0.50	**0.50**	1.00		4.70

[#] HCD Historical control data based on 39 cytogenicity tests performed in the laboratory from December 1988 to December 1994

Table B.6.4.1.27-3: *In vitro* cytogenicity test ([redacted]1995), 6 h incubation with and without metabolic activation

Compound	Concentration [µg/mL]	No. of metaphases scored	Genotoxicity						Mitotic index [%]
			no. structural aberrant cells[x]		% structural aberrant cells		PI (%)	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**	Polyploid cells		

MI mitotic index: number of cells in mitosis/ number of cells, based on 1000 cells per culture
PI: polyploid index: (number of polyploid + endoreduplicated cells)/number of cells in mitosis, based on 200 metaphases
MMC Mitomycin C, positive control without S9 mix; B(a)P: Benzo(a)pyrene, positive control with S9 mix

[b] The color of the culture medium turned yellow after addition of the test substance

[c] No chromosome preparations due to cytotoxicity

[x] Total number of chromosome aberrations from 200 metaphases scored

III. CONCLUSION:

In the chromosome aberration test, there was no significant increase in the frequencies of abnormal metaphases with structural chromosome aberrations or polyploid metaphases in the treated groups compared to the control group whatever were the tested concentrations. Based on the results obtained, it was concluded that, under the conditions of this study, the test substance HR-001 did not induce chromosome aberrations in Chinese hamster CHL cells with or without the metabolic activation system.

Assessment and conclusion by applicant:
Negative for cytogenicity in Chinese hamster lung cells with and without metabolic activation.
The study was performed under GLP conditions and in accordance with OECD guideline 473 (1983). There were only minor deviations when compared to the currently valid OECD 473 (2016). The number of metaphases was only 200, which was the number to be investigated recommended by the previous OCED 473 (1997). In addition, cytotoxicity was evaluated based on mitotic indices instead of measuring the relative population doubling or the relative increase in cell count.

Historical control data were not provided and evaluation criteria of the test were inconsistent with those specified in the current guideline which requires that for a positive response: a. at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, b. the increase is dose-related when evaluated with an appropriate trend test, c. any of the results are outside the distribution of the historical negative control data (e.g. Poisson based 95 % control limits).

However, as the increase in the frequencies of abnormal metaphases with structural chromosome aberrations or polyploid metaphases in the treated groups were similar to those of the negative control, the deviations were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for cytogenicity in Chinese hamster lung cells in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.28. *In vitro studies – chromosome aberration, study 4*

Data point	CA 5.4.1/028
Report author	[redacted]
Report year	1995

Report title	Evaluation of the ability of glyfosaat to induce chromosome aberrations in cultured peripheral human lymphocytes (with independent repeat)
Report No	141918
Document No	Not reported
Guidelines followed in study	OECD 473 (1983), EEC Directive 92/69
Deviations from current test guideline OECD 473 (2016)	Only 200 cells in metaphase were evaluated, whereas the currently valid OECD 473 (2016) recommends the evaluation of 300 metaphase cells per condition. A short-term exposure in the absence of metabolic activation as recommended by OECD 473 (2016) was not included. There was no historical control data provided for the positive control compounds and historical control data obtained in the testing laboratory were not provided. pH measurements were not performed. Acceptance and evaluation criteria specified in the testing protocol differed from those recommended by OECD 473 (2016).
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

A chromosome aberration test in human peripheral lymphocytes was conducted to investigate the potential of glyphosate (batch: 22021, purity: 96 %) to induce chromosomal aberrations *in vitro* in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Concentrations were selected based on the results of a preliminary cytotoxicity test, in which a reduction of the mitotic index of ≥ 50 % was observed at 333 µg/mL in the presence of S9 mix and at 333 and 1000 µg/mL in the absence of S9 mix (24- and 48-hours sampling time point).

Two independent experiments were performed. In each experiment, corresponding solvent (DMSO) and positive controls (mitomycin C in the absence of S9 mix and cyclophosphamide in the presence of S9 mix) were included.

In the first experiment, the cultures were treated for 3 hours in the presence of S9 mix at concentrations in the range of 33 - 562 µg/mL, for 24 hours in the absence of S9 mix with concentrations in the range of 33 - 237 µg/mL and for 48 hours in the absence of S9 mix with concentrations in the range of 56 – 333 µg/mL. The cells treated in the presence of S9 mix were sampled 24 and 48 hours after start of exposure, whereas the cells treated in the absence of S9 mix were prepared directly following end of exposure.

In the second experiment, the cultures were exposed for 3 hours in the presence of S9 mix at concentrations in the range of 100 – 562 µg/mL and for 24 hours in the absence of S9 mix at concentrations in the range of 33 – 333 µg/mL. Chromosome preparations in the second experiment were made 24 hours after start of treatment only.

In each experiment, a total of 200 metaphases per condition was scored for structural and numerical chromosome aberrations. Cytotoxicity was assessed as mitotic index (MI) and evaluated for 1000 cells per culture.

Precipitation of the test item in culture medium was noted at concentrations of 562 µg/mL and above, both in the presence and absence of S9 mix. Cytotoxicity, evident as a reduction in the mitotic index of ≥ 50 %, was observed for the 24-hours sampling time point in the absence of metabolic activation at ≥ 133 µg/mL (first experiment) and for the 24-hours sampling time point in the absence of metabolic activation at 178 and 333 µg/mL (second experiment). Cytotoxicity was further observed after treatment for 48 hours in the absence of metabolic activation and after treatment for 3 hours in the presence of metabolic activation (24-hours sampling time point), however, the mitotic indices were reduced by < 50 %.

After treatment with glyphosate, there was no statistically significant increase in the number of cells with chromosomal aberrations observed in any experiment at any concentration when compared to solvent controls, neither in the presence nor in the absence of metabolic activation.
In addition, there was no increase in the frequencies of polyploid metaphases in any of the tested conditions with or without S9 mix.

The number of aberrant metaphases found in the solvent control cultures were within the range of the laboratory's historical control data. The positive controls mitomycin C and cyclophosphamide produced statistically significant increases in the frequency of aberrant cells, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Based on the experimental findings there is no evidence for a clastogenic potential for glyphosate in peripheral human lymphocytes, neither in the presence nor in the absence of metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

	Glyfosaat
Identification:	Not specified
Description:	White powder
Lot/Batch #:	22021
Purity:	96 %
Stability of test compound:	The stability of the test item under storage conditions (at room temperature in the dark) was guaranteed until the mentioned expiry date 01 Jan 1998. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	Dimethylsulfoxide (DMSO), final concentration 0.9 % (v/v)

2. Control Materials:

Negative control:	A negative control was not employed in this study.
Solvent (vehicle) control:	DMSO (0.9 % (v/v) final concentration in medium)
Positive control:	-S9 mix: Mitomycin C (MMC): 0.1 and 0.2 µg/mL in Hank's Balanced Salt solution (HBSS) for 24- and 48-hours sampling, respectively +S9 mix: Cyclophosphamide (CP): 15 µg/mL in Hank's Balanced Salt solution (HBSS)

3. Metabolic activation:

S9 mix was routinely prepared from the livers of adult male Wistar rats, which received a single intraperitoneal injection of 500 mg/kg bw Aroclor 1254. Five days after treatment, the animals were sacrificed and the S9 homogenates were isolated. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factor.

S9 mix component	Concentration	Unit
HEPES	4.0	mM
KCl	33.0	mM
NADPH-generating system		
Glucose 6-phosphate	6.5	mM
NADP	4.6	mM
$MgCl_2$	5.0	mM
S9	50	% (v/v)

4. Test organism:

Human peripheral blood was obtained by venepuncture from a healthy, male volunteer and collected in heparinised vessels.

5. Cell culture:

Medium: Ham's F10 medium without thymidine and hypoxanthine, supplemented with 20 % (v/v) fetal calf serum, 2 mM L-glutamine, penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively), 1.2 g/L sodium bicarbonate and 30 U/mL heparin.

Incubation:	*In a humidified atmosphere (80 – 95 %) containing 5 % CO_2 in the dark.*
Cell culture establishment prior to exposure	*0.5 mL of whole blood was cultured with 5 mL culture medium and 0.1 mL of 9 mg/mL phytohaemagglutinin for 48 hours prior to treatment.*

2. Test concentrations and number of replicates:

(a) Preliminary cytotoxicity assay

Metabolic activation	Duration of exposure (Fixation time)	Concentrations	Replicates
-S9 mix	24 h (24 h)	10, 33, 100, 333 and 1000 µg/mL	Single culture
-S9 mix	48 h (48 h)	10, 33, 100, 333 and 1000 µg/mL	Single culture
+S9 mix	3 h (24 h)	10, 33, 100, 333 and 1000 µg/mL	Single culture

(b) Main cytogenicity test:

Metabolic activation	Duration of exposure (Fixation time)	Concentrations	Replicates
First experiment			
-S9 mix	24 h (24 h)	33*, 56, 100*, 133, 178 and 237* µg/mL	Duplicate
-S9 mix	48 h (48 h)	56, 100, 133, 178, 237* and 333 µg/mL	Duplicate
+S9 mix	3 h (24 h)	33, 100, 133, 178, 237*, 333* and 562* µg/mL	Duplicate
+S9 mix	3 h (48 h)	33, 100, 133, 178, 237, 333 and 562* µg/mL	Duplicate
Second experiment			
-S9 mix	24 h (24 h)	33*, 100, 133, 178, 237* and 333* µg/mL	Duplicate
+S9 mix	3 h (24 h)	100, 333*, 422* and 562* µg/mL	Duplicate

* Samples analysed for chromosomal aberrations

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 15 Mar – 28 May 1995
Finalisation date: 30 Jun 1995

2. Preliminary cytotoxicity test:
In a preliminary cytotoxicity test, human lymphocytes were treated with the test item at concentrations of 10 to 1000 µg/mL both, with and without metabolic activation under the same conditions as in the main mutagenicity test (described below). The highest concentration was selected based on the solubility of the test substance in culture medium. One single cell culture per condition was exposed to the test item for 3 hours in the presence of S9 mix or for 24 and 48 hours in the absence of S9 mix. Cells treated in the presence of S9 mix were prepared about 24 hours after start of exposure while cells treated in the absence of S9 mix were prepared immediately following end of exposure. Slides were prepared and the mitotic index of each culture was determined by counting the number of 1000 metaphases per culture.

Based on the results of the preliminary test, the concentrations for the main cytogenicity assay were selected. The concentrations of the test substance that was expected to show clear indication of toxicity with a mitotic index of ≥ 50 % was selected for scoring of chromosome aberrations.

3. Main cytogenicity test:

Treatment: Following cell culture establishment, duplicate cultures per condition were exposed to the test item, solvent or positive control in the presence and absence of metabolic activation. Two independent experiments were performed. Corresponding solvent (DMSO) and positive controls (mitomycin C in the absence of S9 mix and cyclophosphamide in the presence of S9 mix) were included in each experiment.
In the first experiment, the cultures were treated for 3 hours in the presence of S9 mix at concentrations in the range of 33 - 562 µg/mL, for 24 hours in the absence of S9 mix with concentrations in the range of 33 - 237 µg/mL and for 48 hours in the absence of S9 mix with concentrations in the range of 56 – 333 µg/mL. The cells treated in the presence of S9 mix were prepared for chromosome analysis 24 and 48 hours after start of exposure. After 3 hours of exposure with S9 mix, the cells were rinsed once with Hank's buffered salt solution (HBSS) to remove the test compound, re-suspended in medium and incubated for another 20 – 22 hours (24-hours fixation time) or for 44 - 46 hours (48-hours fixation time). The cells treated in the absence of S9 mix were prepared directly following end of exposure.
In the second experiment, the cultures were exposed for 3 hours in the presence of S9 mix at concentrations in the range of 100 – 562 µg/mL and for 24 hours in the absence of S9 mix at concentrations in the range of 33 – 333 µg/mL. Chromosome preparations in the second experiment were made 24 hours after start of treatment only.

Spindle inhibition: During the last 3 hours of the culture period, cell division was arrested by addition of 0.5 µg/mL colchicine (0.5 µg/mL) to each culture.

Cell harvest: The cell cultures were centrifuged and the remaining cell pellet swollen in 0.56 % hypotonic potassium chloride solution for 5 minutes at 37 °C. Afterwards, the cells were fixed with 3 changes of methanol : acetic acid fixative (3:1 v/v).

Slide preparation: Fixed cells were dropped on glass slides which were immersed for 24 hours in a 1:1 mixture of 96 % (v/v) ethanol/ether and cleaned with a tissue. Two slides were prepared per culture. Slides were allowed to dry, stained for 10 – 30 minutes with 5 % (v/v) Giemsa solution, rinsed with tap water, allowed to dry again and cleared in xylene before embedding in DePeX and mounting.

Metaphase analysis A total number of 200 metaphase cells per condition (100 metaphase cells per culture) were examined by light microscopy. In case the number of aberrant cells (gaps excluded) was ≥ 25 in 50 metaphases no more metaphases were examined. Only metaphases containing 46 chromosomes were analysed. The following data were recorded:

- The number of cells with chromosomal aberrations.

The number and type of structural chromosomal aberrations, Chromosome gaps, -breaks, -exchanges, -intrachanges, chromatid gaps, -breaks and -deletions, centromere state, ring chromosomes, and other structural chromosome aberrations such as multiple aberration.

- Numerical variations such as endoreduplication and polyploidy.

Cytotoxicity: The mitotic index of each culture was calculated based on the number of cells in metaphase observed per 1000 cells scored.

4. Statistics

The number of cells with chromosomal aberrations at each dose were compared with those of corresponding solvent controls and statistically evaluated using a chi-square test

5. Acceptance criteria

The chromosome aberration test was considered acceptable if the following criteria were met:

- The numbers of chromosome aberrations found in the solvent control cultures should reasonably be within the laboratories historical control data range.
- The positive control substances should produce a statistically significant increase in the number of cells with chromosomal aberrations.

6. Evaluation criteria

A test substance was considered positive (clastogenic) in the chromosome aberration test if the following criteria were met:

- It induced a dose-related statistically significant increase in the number of cells with chromosome aberrations.
- A statistically significant increase in the frequency of aberrations was observed in the absence of a clear dose-response relationship.

A test substance was considered negative (not clastogenic) in the chromosome aberration test if none of the tested concentrations induced a statistically significant increase in the number of cells with chromosomal aberrations.

II. RESULTS AND DISCUSSION

A. ANALYTCAL DETERMINATIONS

Analytical determinations of the test substance in the solvent have not been performed, as not required by the test guideline.

B. CYTOTOXICITY

A detailed description on cytotoxic effects at individual concentrations was not provided in the study report. Description of cytotoxicity in the following section is based on re-evaluation of the raw data provided in the study report. A cytotoxic effect was considered evident when a reduction of the mitotic index of ≥ 50 % was observed.

Preliminary cytotoxicity test

In the preliminary cytotoxicity test, cytotoxicity was observed at 333 µg/mL in the presence of S9 mix and at 333 and 1000 µg/mL in the absence of S9 mix (24- and 48-hours sampling time point). For the 24-hours sampling time point at 333 and 1000 µg/mL in the absence of S9 mix and for the 48-hours sampling time point in the absence of S9 mix no metaphase cells at all were detected.

The results obtained in the preliminary cytotoxicity test were used to select concentrations for the main mutagenicity assay. The highest test compound concentration was expected to reduce mitotic index by ≥ 50 % compared to corresponding solvent controls. Therefore, for the main cytogenetic test concentrations of 237 and 333 µg/mL were selected as highest concentrations in the absence of S9 mix (24- and 48-hours sampling time point, respectively) and 562 µg/mL was chosen as highest concentration in the presence of S9 mix.

Main mutagenicity test

In the main mutagenicity test, cytotoxicity evident as a reduced mitotic index of ≥ 50 % was observed for the 24-hours sampling time point in the absence of metabolic activation at ≥ 133 µg/mL (first experiment) and for the 24-hours sampling time point in the absence of metabolic activation at 178 and 333 µg/mL (second experiment). There was also evidence of toxicity for the 48-hours sampling time point in the absence of metabolic activation (first experiment) and for the 24-hours sampling time point in the presence of metabolic activation (second experiment), but the mitotic indices were < 50 (please refer to Table B.6.4.1.28-1).

C. SOLUBILITY

Precipitation of the test item was reported in the preliminary cytotoxicity test as well as the main cytogenicity test, at 562 µg/mL and at higher concentrations, both in the presence and absence of S9 mix.

D. CYTOGENICITY

There was no statistically significant increase in the number of cells with chromosomal aberrations observed in any experiment at any concentration when compared to solvent controls, neither in the presence nor in the absence of metabolic activation.

In addition, there was no increase in the frequencies of polyploid metaphases in any of the tested conditions with or without S9 mix.

The number of aberrant metaphases found in the solvent control cultures were within the range of the laboratory's historical control data. The positive controls mitomycin C and cyclophosphamide produced statistically significant increases in the frequency of aberrant cells, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Table B.6.4.1.28-1: Evaluation of the ability of glyfosaat to induce chromosome aberrations in cultured peripheral human lymphocytes ([redacted], 1995), 24 and 48 h exposure (-S9), 3 h exposure (+S9)

Compound	Conc. [µg/mL]	No. of metaphases scored	Genotoxicity								MI [%]
			no. structural aberrant cells		% structural aberrant cells				PI	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**			Polyploid cells		
Without metabolic activation; 24-hours treatment and sampling											
Solvent (DMSO)	0	200	4.0	**3.0**	2.0	**1.5**			2	negative	100
HCD[#] mean ± SD	0	100	1.0 ± 1.1								
Test item	33	200	4.0	**2.0**	2.0	**1.0**			2	negative	96
	100	200	2.0	**1.0**	1.0	**0.5**			0	negative	78
	237	200	5.0	**3.0**	2.5	**1.5**			1	negative	47
MMC	0.2	200	71.0*	**50.0***	35.5*	**25.0***			0	positive	44
Without metabolic activation; 48-hours treatment and sampling											
Solvent (DMSO)		200	4.0	**1.0**	2.0	**1.0**			0	negative	100
HCD[#] mean ± SD	0	100	1.0 ± 1.1								
Test item	237	200	1.0	**0.0**	0.5	**0.0**			1	negative	65
MMC	0.1	200	81.0***	**67.0***	40.5*	**33.5***			0	positive	83
With metabolic activation; 3-hours treatment, 24 h sampling time											
Solvent (DMSO)		200	6.0	**4.0**	3.0	**2.0**			1	negative	100
HCD[#] mean ± SD	0	100	0.8 ± 0.9								
Test item	237	200	2.0	**0.0**	1.0	**0.0**			0	negative	101
	333	200	3.0	**2.0**	1.5	**1.0**			2	negative	89
	562	200	4.0	**4.0**	2.0	**2.0**			1	negative	55
CP	15	200	89.0*	**53.0***	44.5*	**26.5***			1	positive	33
With metabolic activation; 3-hours treatment, 48 h sampling time											
Solvent (DMSO)		200	1.0	**0.0**	0.5	**0.0**			0	negative	100
HCD[#] mean ± SD	0	100	0.8 ± 0.9								
Test item	562	200	0.0	**0.0**	0.0	**0.0**			0	negative	121

[#] HCD Historical control data from the laboratory's historical control range (time period for data generation not specified); MI Mitotic index

MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix

* $p < 0.001$ with Chi-square test

Table B.6.4.1.28-2: Evaluation of the ability of glyfosaat to induce chromosome aberrations in cultured peripheral human lymphocytes ([redacted], 1995), 24 h exposure (-S9), 3 h exposure (+ S9)

Compound	Conc. [µg/mL]	No. of metaphases scored	Genotoxicity								MI [%]
			no. structural aberrant cells		% structural aberrant cells				PI	Judge	
			incl. gaps	**excl. gaps**	incl. gaps	**excl. gaps**			Polyploid cells		
Without metabolic activation; 24-hours treatment and sampling											
Solvent (DMSO)	0	200	1.0	**0.0**	0.5	**0.0**			0	negative	100
HCD[#] mean ± SD	0	100	1.0 ± 1.1								
Test item	33	200	2.0	**0.0**	1.0	**0.0**			0	negative	84
	237	200	5.0	**3.0**	2.5	**1.5**			0	negative	61
	333	200	4.0	**2.0**	2.0	**1.0**			0	negative	34
MMC	0.2	200	51.0*	**51.0***	25.5*	**25.5***			0	positive	43
With metabolic activation; 3-hour treatment, 24 h sampling time											
Solvent (DMSO)		200	4.0	**3.0**	2.0	**1.5**			0	negative	100
HCD[#] mean ± SD	0	100	0.8 ± 0.9								
Test item	333	200	7.0	**5.0**	3.5	**2.5**			1	negative	93
	422	200	4.0	**2.0**	2.0	**1.0**			0	negative	78
	562	200	3.0	**1.0**	1.5	**0.5**			0	negative	85
CP	15	200	53.0*	**53.0***	26.5*	**26.0***			0	positive	36

[#] HCD Historical control data from the laboratory's historical control range (time period for data generation not specified); MI Mitotic index

MMC Mitomycin C, positive control without S9 mix; CP:cyclophosphamide, positive control with S9 mix

* $p < 0.001$ with Chi-square test

III. CONCLUSION:

Based on the experimental findings there is no evidence for a clastogenic potential for glyphosate in peripheral human lymphocytes, neither in the presence nor in the absence of metabolic activation.

Assessment and conclusion by applicant:
Negative for cytogenicity (Chromosome Aberration test) in peripheral human lymphocytes with and without metabolic activation.

The study was performed under GLP conditions and in accordance with OECD guideline 473 (1983). When compared with the currently valid OECD guideline 473 (2016), several deviations became evident. The dose levels investigated were rather low when compared to other studies that were provided for the evaluation of glyphosate. In addition, a short-term exposure in the absence of S9 mix was not included in the experiment. Accordingly, the study was considered to provide supporting information. Further guideline deviations were considered to not impact the validity of the study.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for cytogenicity (Chromosome Aberration test) in peripheral human lymphocytes in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.29. *In vitro studies – chromosome aberration, study 5*

Data point:	CA 5.4.1/029
Report author	[redacted]
Report year	1989
Report title	Report on the possible chromosome damaging effect of glyphosate in Chinese hamster ovary cells
Report No	Not reported
Document No	Not reported
Guidelines followed in study	The study was conducted according to Natarajan et al.[4,] (1967), according to guidelines and recommendations of the United Kingdom Environmental Mutagen Society (UKEMS) and similar to OECD 473 (2016).
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate (batch: 978, purity: not specified) was assessed for its ability to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells *in vitro*. The test was conducted in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Glyphosate concentrations were selected based on the results of a preliminary toxicity test, in which cytotoxicity was observed at ≥ 2500 µg/mL in the presence of S9 mix and at ≥ 1250 µg/mL in the absence of S9 mix. Two independent experiments were performed. In each experiment, solvent (medium) and positive controls (ethylmethane sulfonate, 1 µg/mL without S9 mix and cyclophosphamide, 25 µg/mL with S9 mix were included. Tested glyphosate concentrations were 250 – 1000 µg/mL in the absence of S9 mix and 62.5 – 250 µg/mL in the presence of S9 mix. In both experiments, the cells were exposed for 3 hours in the presence and absence of S9 mix, followed by two washing steps and re-incubation for further 18 hours[5]. Chromosomes were prepared about 21 hours after start of exposure[5]. In each experiment, a total of 200 metaphases per condition was scored for structural chromosome and chromatid aberrations.
Short description of results:	Precipitation of the test item in culture medium was not reported. Cytotoxicity was not monitored in the cytogenicity experiments, as the concentrations selected were chosen to be non-cytotoxic. In the first experiment, there was a mathematically significant increase in the incidence of aberrant cells and the incidence of total chromosome aberrations at 62.5 µg/mL in the presence of metabolic activation. As the effect was noted at the lowest concentration and as a dose-dependency was lacking, the observation was considered incidental. In the second experiment, there was no statistically

[4] Natarajan, A.T., Tates, A.B., van Buul, P.W., Meijery, M. and De Vogel, N.: Cytogenic effects of mutagens/carcinogens after activation in microsomal system in vitro. I. Induction of chromosome aberration and sister chromatid exchange by diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) in CHO cells in the presence of rat liver microsome. Mut. Res., 37, 83-90 (1976)

[5]: There are inconsistencies in the study report regarding the duration of exposure (2 or 3 hours) and the duration of post-exposure incubation (16 - 18 hours incubation followed by 2 hours incubation with colchicine or 16 hours incubation in total) prior to metaphase analysis. It was assumed that the cells were exposed for 3 hours, followed by 18 hours of incubation (including 2 hours of treatment with colchicine) and chromosome preparation 21 hours after start of exposure.

	significant increase in the number of cells with chromosomal aberrations or the total number of aberrations at any concentration when compared to solvent controls, neither in the presence, nor in the absence of metabolic activation. The positive controls induced statistically significant aberrations in both experiments, demonstrating the functionality of the metabolic activation system and the sensitivity of the test. Based on the experimental findings and under the conditions of the test, glyphosate has no clastogenic potential in CHO cells *in vitro*, neither in the presence nor in the absence of metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	The study is considered not acceptable due to the large number of deviations when compared to the currently valid OECD guideline 473 (2016). Only 100 – 200 metaphase cells were evaluated and only structural chromosome aberrations but no numerical aberrations were considered. In addition, the maximum concentration did not meet the cytotoxicity recommendations specified in the current guideline. Cytotoxicity was not measured in the main cytogenicity test, but evaluated only in a preliminary toxicity test based on cell morphology. Besides a number of further guideline deviations, there were inconsistencies regarding the duration of exposure and reporting deficiencies regarding the source and purity of the test material. The study was therefore considered not valid.
Reasons why the study report is not available for submission	**Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation due to the large number of deviations when compared to the currently valid OECD guideline 473 (2016).

B.6.4.1.30. *In vitro studies – mammalian gene mutation, study 1*

Data point	CA 5.4.1/030
Report author	[redacted]
Report year	1998
Report title	Glyphosate Acid: L5178Y TK$^{+/-}$ Mouse Lymphoma Gene Mutation Assay
Report No	CTL/P/4991
Document No	Not reported
Guidelines followed in study	OECD 476 (1984), US EPA OPPTS 870.5300 (1998), Council Directive 2000/32/EEC B.17 (2000)
Deviations from current test guideline OECD 490 (2016)	The newly introduced cytotoxicity parameters RTG (relative total growth), SG (suspension growth) and RSG (relative suspension growth) could not be re-calculated, since no data on suspension growth were available. In the present study, cytotoxicity was evaluated based on cloning efficiency, in accordance with the previous guideline version. Historical control data were only provided for negative/vehicle controls but not for the positive control. pH changes observed upon compound addition were not buffered. Acceptance and evaluation criteria were inconsistent with those specified in OECD 490 (2016). The deviations are not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate acid (batch: P24, purity: 95.6 %) was tested *in vitro* for its ability to induce forward mutations in

mammalian cells by assessing the mutation of the TK locus in Mouse Lymphoma L5178Y $TK^{+/-}$ cells. Two independent experiments with a treatment duration of 4 hours were conducted in the presence or absence of metabolic activation (phenobarbital/ ß-naphthaflavone induced rat liver S9 fraction) using two parallel cultures each. Based on the results of a preliminary range-finding experiment, in which the pH value was excessively reduced at a concentration of 2000 µg/mL, concentrations in the range of 296 to 1000 µg/mL were used in the main mutation assay (1500 µg/mL included in experiment I, but not evaluated for mutagenicity; 296 µg/mL included in experiment II). Solvent controls and appropriate positive controls (ethylmethane sulphonate (EMS), without S9 mix and n-nitrosodimethylamine (NDMA), with S9 mix) were included in each experiment.

After 4 hours of exposure, the cells were incubated for 48 hours to allow expression of the mutant phenotype. The expression period was followed by a selection period, where cells were incubated in selection medium containing trifluorothymidine (TFT) for 10-13 days.

Precipitation of the test substance was not reported. A reduction in pH of the treatment medium was observed at 1000 µg/mL and above, however, only little cytotoxicity was observed at the concentrations tested, both in the presence and in the absence of metabolic activation. A biologically relevant and dose-dependent increase in the mean number of mutant colonies was not observed up to the highest test item dose, independent of the presence or absence of metabolic activation. Appropriate reference mutagens (EMS and NDMA) used as positive controls showed a distinct increase in induced mutant colonies, indicating the test-system to be sensitive and valid. The negative controls gave mutant frequencies within the range of historical control data.

Based on the results of the present study, glyphosate acid did not induce forward mutations in the mouse lymphoma assay with L5178Y $TK^{+/-}$ cells in the absence or presence of metabolic activation. Thus, the test item is considered not mutagenic in mammalian cells *in vitro*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate acid

Identification:	
Description:	White solid
Lot/Batch number:	P24
Purity:	95.6 % (w/w)
Stability of test compound:	The stability of the test item at storage conditions (in an anti-static bag at ambient temperature) and in solvent (vehicle) were not specified.

2. Control material:

Negative control:	A negative control was not employed in this study
Solvent (vehicle) control:	Dimethylsulfoxide (DMSO), final concentration 1 %
Positive control:	- S9 mix: Ethylmethane sulphonate (EMS), 750 µg/mL in DMSO + S9 mix N-nitrosodimethylamine (NDMA), 600 µg/mL in DMSO

3. Metabolic activation:

S9 mix was prepared from the livers of male Sprague-Dawley rats, that received oral doses of phenobarbital (80 mg/kg bw) and ß-naphthaflavone (100 mg/kg bw) on three consecutive days. The treated animals were sacrificed on the day following the last dose. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factors. The co-factor solution was prepared as a stock solution of 75 mM NADP (disodium salt) and 1200 mM glucose-6-phosphate (monosodium salt) in RPMI 1640 culture medium with a final pH adjusted to 7.5. S9 fraction was added at 5 % (1 mL S9 added to the 20 mL cell culture) and co-factors at 1 % (200 µL to the 20 mL cell culture).

S9 mix component	**Concentration**	**Unit**
RPMI 1640 culture medium (pH 7.5)		
NADPH-generating system		
Glucose 6-phosphate Na salt	12	mM
NADP Na_2 salt	0.75	mM
S9	5	% (v/v)

4. Test organism:

L5178Y $TK^{+/-}$ mouse lymphoma cells were used. Stocks were maintained in liquid nitrogen and thawed immediately before use. Each batch used for mutagenicity testing was screened for mycoplasma contamination.

5. Cell culture media:

Cultivation medium: RPMI 1640 medium with Hepes, supplemented with 4 mM L-glutamine, 200 IU/mL penicillin and 200 µg/mL streptomycin

Growth medium: Cultivation medium supplemented with 10 % horse serum (in case of microwell cultivation 20 % horse serum

Treatment medium (± S9:) Cultivation medium supplemented with 5 % horse serum

Selection medium: Growth medium supplemented with 20 % horse serum and 4 µg/mL trifluorothymidine (TFT)

Incubation: At 37 °C, 5 % CO_2 and 98 % humidity

6. Locus examined Thymidine kinase (TK)

7. Test concentrations and number of replicates:

(a) Preliminary range-finding assay

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	4 h	125, 250, 296, 444, 500, 667, 1000, 1500 and 2000 µg/mL	Duplicate
+S9 mix	4 h	125, 250, 296, 444, 500, 667, 1000, 1500 and 2000 µg/mL	Duplicate

(b) Main mutation assay:

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	4 h	296*, 444*, 667*, 1000* and 1500 µg/mL	Duplicate
+S9 mix	4 h	296*, 444*, 667*, 1000* and 1500 µg/mL	Duplicate

* Samples evaluated for mutagenicity

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 22 Nov 1995 – 12 Mar 1996
Finalisation date: 24 May 1996

2. Preliminary cytotoxicity test:
A preliminary dose-range finding study was performed to determine the concentrations of the test item to be used in the main mutation assay. Toxicity was evaluated based on changes in the pH of the treatment medium and on osmolality. Data of the range-finding test were not included in the study report. Based on the observations in pH changes during the preliminary toxicity experiment, concentrations for the mutation assay were selected.

3. Main mutation assay:
Pre-treatment of cells:
A bulk cell culture with a density in the range of 1 - 1.2 x 10^6 cells per mL was prepared prior to each experiment. The culture was then diluted 50:50 (v/v) with serum-free medium to obtain a reduced serum content of 5 % at treatment time. Each 20 mL treatment culture (5 - 6 x 10^5) cells were used from the bulk culture for each test condition.

Treatment:

Just prior to treatment, the medium was supplemented with S9 mix in the appropriate cell cultures with metabolic activation. Two cultures were treated in parallel for each group. Aliquots of the test substance, solvent or positive control were added and the cultures were exposed for 4 hours, rotating on a roller apparatus at 37 °C. At the end of the treatment period, the cells were centrifuged and the cell pellets were re-suspended in 50 mL fresh culture medium.

Expression period:
After treatment, all cell cultures were incubated for a two days expression period. To maintain exponential growth during the expression time, each culture was counted daily and the cell numbers were adjusted to 2 x 10^5 cells/mL in 50 mL medium, thereby ensuring approximately 10^7 cells at each subculture.
After 48 hours expression period, the cell density of each culture was determined and each culture was divided into two series of dilutions. The first was used for the selection of mutants, the second was to assess the viability of the cultures in the absence of selection medium.

Selection period:
For the selection of mutants, each post-expression culture was diluted to give 50 mL at 1 x 10^4 cells/mL. Trifluorothymidine (TFT) was added and each culture was then dispensed at 200 µL per well into two 96 well microtiter plates (2000 cells/well). After incubation for 10 - 13 days cell growth was assessed. All plates were scored for the number of negative wells (no cell growth) and the number of wells containing small or large colonies. Small colonies were considered to be associated with clastogenic effects, large colonies were considered to be associated with gene mutation effects.

4. Cytotoxicity:

Cloning efficiency CE_1 (survival)
At the end of the exposure period, a sample of each cell culture was collected to assess survival. The cultures were diluted to give 50 mL at 8 cells/mL and dispensed at 200 µL/well into two 96-well plates (1.6 cells/well). After an incubation of 10 - 13 days the plates were scored for empty wells.

Cloning efficiency CE_2 (viability)
After the expression period, 2 days after end of exposure, the cultures were divided into two series of dilutions. The first was used for the selection of mutants, the second was to assess the viability of the cultures in the absence of selection medium. A sample from each mutation culture was diluted to give 50 mL at 8 cells/mL and dispensed at 200 µL per well into two 96 well microtiter plates (1.6 cells/well). After incubation for 10 - 13 days the plates were scored for empty wells.

5. Evaluation:

Cytotoxicity (cloning efficiency CE)
The number of empty wells of the two seeded 96-well plates was scored and recorded.

CE_1 (survival)
The cytotoxicity of the test substance after the exposure period was determined for each test group and is indicated as absolute and relative cloning efficiency (CE_1 and RCE_1, respectively).

CE_2 (viability)
The cytotoxicity of the test substance at the end of the expression period was determined for each test group and is given as absolute and relative cloning efficiency (CE_2 and RCE_2, respectively).

The cloning efficiency (CE, %) was calculated for each test group as follows:

$$\mathrm{CE_x} = \frac{-\ln \frac{\text{total number of empty wells}}{\text{total number of seeded wells (96)}}}{\text{number of seeded cells per well (1.6)}} \times 100$$

$$\mathrm{RCE_x} = \frac{\mathrm{CE_x} \text{ of the test group}}{\mathrm{CE_x} \text{ of the negative or vehicle control}} \times 100$$

Mutant frequency (MF)
The number of empty wells and the number of wells containing colonies were scored and reported. The colonies are classified into large colonies (indication of gene mutation) and small colonies (indication of chromosome

breakage). Large colonies are defined as more than 25 % of the diameter of the well. A large colony should have shown less densely packed cells, especially around the edges of the well. Small colonies are defined as less than 25 % of the diameter of the well. A small colony should have shown a dense clonal morphology. Any well which contained more than one small colony was scored as a small colony. Any well which contained more than one large colony was scored as a large colony. Any well which contained a combination of large and small colonies was scored as a large colony. An empty well was one which contained no cell growth.

Uncorrected mutant frequency:
The uncorrected mutant frequency per 10^6 cells (MF_{uncorr}) was calculated for each test group as follows:

$$MF_{uncorr.} = \frac{-\ln \frac{\text{total number of empty wells}}{\text{total number of seeded wells (96)}}}{\text{number of seeded cells per well (2000)}} \times 10^6$$

Corrected mutant frequency
The corrected mutation frequency (MF_{corr}) was calculated regarding the values of CE_2:

$$\text{MF corrected} = \frac{\text{MF uncorrected}}{CE_2} \times 100$$

Determination of borderline mutant frequency based on GEF
The GEF (global evaluation factor) method requires that the MF exceeds a value based on the global distribution of the background MF of the test method. This value is defined as the mean of the negative/vehicle MF distribution plus one standard deviation.
Based on a large data base (n = 493 experiments) from six laboratories a GEF of 126 mutant colonies per 10^6 cells [mean $MF_{corr} = 99 \times 10^{-6}$ colonies; standard deviation = 27×10^{-6} colonies] was calculated for the microwell method. To be judged positive, the mutation frequency has to exceed a threshold of 126 colonies per 10^6 cells (GEF) above the concurrent negative/vehicle control value. The borderline mutant frequency was calculated for each experiment separately as follows:
Borderline MF = $MF_{vehicle\ control\ corr}$ + GEF (126×10^{-6})
The borderline MF was not evaluated as part of the present study, but was determined retrospectively for this evaluation.

6. Statistics:
Statistical analysis was not performed in the study.

7. Acceptance criteria:
The assay was considered valid if the following criteria were met:

- Post-expression cloning efficiencies of 50 % or greater were achieved for the solvent control viability plates.
- Spontaneous mutant frequencies of the solvent controls were within the range of the laboratories historical control data.
- Results obtained with the positive controls gave an unequivocal positive response in the presence and absence of S9 mix.

8. Evaluation criteria:
A substance was considered to be mutagenic if the following criteria are met:

- There was a statistically significant and dose-related increase in mutant frequency, but not only at concentrations eliciting cytotoxicity.
- The increase in mutant frequency was above those of solvent controls and reproducible in an independent experiment.

A substance was considered to be negative for mutagenicity if there was no reproducible statistically significant dose-related increase in mutant frequency observed. When reproducible significant increases in mutant frequency were seen only at levels of excessive toxicity, or when such increases were not accompanied by an increase in absolute numbers of mutants over solvent control values, consideration was given to such factors as statistical significance of the difference between treated and control cultures, and dose response relationships in order to clarify the response. Failing this, results from an independent experiment were obtained to attempt to clarify the result.

II. RESULTS AND DISCUSSION

1. ANALYTICAL DETERMINATIONS

In view of the short-term nature of the study, no analysis of stability, homogeneity or achieved concentrations were carried out on the preparation of the test item or positive control substance formulations either prior to or after addition to the cell cultures. Analytical determinations are not required by the test guideline.

2. CYTOTOXICITY

In the preliminary cytotoxicity test, toxicity was evaluated based on changes in pH of the treatment medium. A concentration of 2000 µg/mL in the presence and absence of S9 mix was found to produce an excessive reduction in the pH of the treatment medium. Based on these findings, a maximum concentration of 1500 µg/mL with and without S9 mix was considered appropriate for the main mutation assay.

In the main mutation assay, 1500 µg/mL was found to produce an excessive reduction in the pH of the treatment medium (0.99 units), whereas reductions in pH seen at 1000 µg/mL (0.59 and 0.40 units in the first and second experiment, respectively) were considered acceptable and not to affect the outcome of the study. Very little cytotoxicity was seen at the concentrations tested.

Table B.6.4.1.30-1: Glyphosate Acid: L5178Y TK+/- Mouse Lymphoma Gene Mutation Assay ([redacted] 1996), first experiment

Test group	Mutagenicity data	Toxicity data				Mutagenicity data
	Corrected Mutant[$] Frequency per 10^6 cells	Cloning efficiency[#] (CE_1 -survival)		Cloning efficiency[#] (CE_2 -viability)		Uncorrected mutant frequency Per 10^4 cells
	total	absolute	relative (RCE_1)	absolute	relative (RCE_2)	
Without metabolic activation; 4-hour exposure period						
DMSO control	111.00	45.56	100.00	68.17	100.00	1.1
MF threshold[§]	237.00					
Test item [µg/mL]						
444.00	60.00	34.31	75.31	44.73	65.62	0.6
667.00	91.00	43.66	95.83	62.18	91.21	1.0
1000.00	105.00	32.70	71.77	79.29	116.31	1.1
1500.00	-	33.55	73.64	-	-	-
EMS 750 µg/mL	1254.00	12.31	27.02	23.68	34.74	12.6
IMF	*1143.00*					
With metabolic activation; 4-hour exposure period						
DMSO control	84.00	90.35	100.00	74.19	100.00	0.9
Test item [µg/mL]						
444.00	79.00	76.46	84.63	84.72	114.19	0.8
667.00	120.00	51.69	57.21	71.01	95.71	1.2
1000.00	128.00	49.86	55.19	107.03	144.26	1.4
1500.00	-	66.43	73.53	-	-	-
NDMA 600 µg/mL	425.00	57.17	63.28	27.87	37.57	4.3
IMF	*341.00*					

MF: mutant frequency

[$] Mutant frequency values for 10^6 cells. Values differ from those mentioned in study report, where the MF were given for 10^4 cells.

IMF: induced mutant frequency, should be $\geq 300 \times 10^{-6}$ for total MF or $\geq 150 \times 10^{-6}$ for small colonies

[§] $MF_{\text{vehicle control corr}}$ + GEF (126×10^{-6}), rounded

[#] Cloning efficiencies were re-calculated according to the formulas specified in OECD 490 (2016) based on raw data provided in study report

Table B.6.4.1.30-2: Glyphosate Acid: L5178Y TK+/- Mouse Lymphoma Gene Mutation Assay ([redacted], 1996), second experiment

Test group	Mutagenicity data	Toxicity data				Mutagenicity data
	Corrected Mutant[$] Frequency per 10^6 cells	Cloning efficiency[#] (CE_1 -survival)		Cloning efficiency[#] (CE_2 -viability)		Uncorrected mutant frequency Per 10^4 cells
	total	absolute	relative (RCE_1)	absolute	relative (RCE_2)	
Without metabolic activation; 4-hour exposure period						
DMSO control	130.00	59.61	100.00	97.65	100.00	1.3
MF threshold[§]	256.00					
Test item [µg/mL]						
296.00	142.00	77.83	130.57	91.86	94.07	1.4
444.00	117.00	61.84	103.74	119.70	122.58	1.2
667.00	128.00	63.44	106.43	104.41	106.92	1.3
1000.00	222.00	91.38	153.30	122.14	125.08	2.3
EMS 750 µg/mL	1211.00	32.58	54.66	61.30	62.78	12.9
IMF	*1081.00*					
With metabolic activation; 4-hour exposure period						
DMSO control	157.00	62.75	100.00	89.24	100.00	1.6
Test item [µg/mL]						
296.00	148.00	79.67	126.96	91.41	102.43	1.5
444.00	160.00	67.00	106.77	91.07	102.05	1.8
667.00	123.00	62.40	99.44	85.41	95.71	1.3
1000.00	177.00	84.95	135.38	66.77	74.82	1.8
NDMA 600 µg/mL	601.00	69.02	109.99	26.89	30.13	6.4
IMF	*444.00*					

MF: mutant frequency

[$] Mutant frequency values for 10^6 cells. Values differ from those mentioned in study report, where the MF were given for 10^4 cells. The RMS has added the mutant frequencies for 10^4 cell as reported in the study report.

IMF: induced mutant frequency, should be $\geq 300 \times 10^{-6}$ for total MF or $\geq 150 \times 10^{-6}$ for small colonies

[§] $MF_{\text{vehicle control corr}}$ + GEF (126×10^{-6}), rounded

[#] Cloning efficiencies were re-calculated according to the formulas specified in OECD 490 (2016) based on raw data provided in study report

3. SOLUBILITY

Precipitation of the test item was not reported. pH measurements revealed a reduction of about >0.40 units at 1000 µg/mL and above. A significant effect on osmolality was not observed.

4. MUTANT FREQUENCY

There was no biologically relevant and reproducible increase in the number of mutant colonies observed upon treatment with the test item in both main experiments, neither in the presence, nor in the absence of metabolic activation. Mutant frequencies obtained for solvent controls remained within the range of the laboratory's historical control data. Exposure to the positive controls EMS and NDMA induced substantial increases in the mutation frequency in all experiments, demonstrating the sensitivity of the test system and the activity of the S9 mix.

III. CONCLUSION:

Glyphosate acid did not induce increased mutant frequencies in L5178Y $TK^{+/-}$ cells in the presence or absence of S9-mix. Based on the results of the present study and under the experimental conditions chosen, the test item is negative for mutagenicity in mammalian cells *in vitro*.

Assessment and conclusion by applicant:

Negative for mutagenicity in L5178Y $TK^{+/-}$ cells with and without metabolic activation.

The study was performed under GLP conditions and in accordance with OECD guideline 476 (1984). A number of deviations were observed when compared to the currently valid OECD guideline 490 (2016). All deviations were considered to be of minor degree and to not compromising the validity of the study. Mutant frequency and toxicity data given in the study report included data from 10^4 cells. For this evaluation, data were calculated retrospectively for 10^6 cells. In addition, the borderline mutant frequency based on GEF was determined retrospectively. The study was considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for mutagenicity in L5178Y $TK^{+/-}$ cells in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.31. *In vitro studies – mammalian gene mutation, study 2*

Data point	CA 5.4.1/031
Report author	[redacted]
Report year	1991
Report title	Mutagenicity test: *In vitro* Mammalian Cell Gene Mutation Test with Glyphosate, batch 206-JaK-25-1
Report No	12325
Document No	Not reported
Guidelines followed in study	OECD 476 (1983), US CFR part 700 (F) §798.5265 (1987)
Deviations from current test guideline OECD 490 (2016)	The newly introduced cytotoxicity parameters RTG (relative total growth), SG (suspension growth) and RSG (relative suspension growth) could not be re-calculated, since no data on suspension growth were available. In the present study, cytotoxicity was evaluated based on cloning efficiency, in accordance with the previous guideline version. Although the growth rates of the cultures were monitored, the data were not provided within the study report. The number of cells treated was below 6×10^6 cells, the number of cells recommended in OECD 490 (2016). No historical control data were provided for the negative and the positive controls. pH measurements were not performed. Acceptance criteria were not defined in the study report and evaluation criteria specified in OECD 490 (2016) were not applied. The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Conclusion GRG: Valid, Category 2a Conclusion AGG: The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: 206-JaK-25-1, purity: 98.6 %) was tested in a Mouse Lymphoma assay for its ability to induce forward mutations in mammalian cells *in vitro*. In two independent experiments, duplicate cultures of

Mouse Lymphoma L5178Y $TK^{+/-}$ cells were exposed to the test item, medium or appropriate positive controls (100 µg/mL ethylnitrosourea (ENU) without S9 mix and 5-10 µg/mL dimethylbenzanthracene (DMBA) with S9 mix), both, in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Based on the results of a preliminary toxicity test, in which no cytotoxicity was observed up to limit concentrations of 5.0 mg/mL glyphosate in the absence of S9 mix or 4.2 mg/mL glyphosate in the presence of S9 mix, the two experiments of the main mutagenicity assay were conducted at the same concentration ranges.

After 3 hours of exposure with S9 mix or 4 hours of exposure without S9 mix, the cells were incubated for 2-3 days to allow expression of the mutant phenotype. The expression period was followed by a selection period, in which the cells were cultivated in selection medium containing trifluorothymidine (TFT) for a period of 10 days. Cell survival and cell viability were assessed as cloning efficiency 1 and cloning efficiency 2 at the end of the exposure period and after the expression period, respectively.

Precipitation of the test item in the medium was not reported and there was no cytotoxicity observed at any of the tested concentrations, neither in the presence nor in the absence of S9 mix. There was no statistically significant increase in the number of mutant colonies observed upon treatment with glyphosate in both experiments at any of the tested concentrations, neither in the presence, nor in the absence of metabolic activation. Mutant frequencies of the medium control cultures were in the expected range.

A clear increase in mutant frequencies was observed for the positive control ENU in the absence of S9 mix, while the positive control DMBA in the presence of S9 mix showed a rather moderate increase in mutant frequency at 5.0 µg/mL. Thus, the second experiment was conducted with a higher DMBA concentration of 10 µg/mL. The higher dose revealed a much stronger response in mutant colony formation, demonstrating the sensitivity of the test system and the activity of the S9 mix.
Based on the results of the present study, glyphosate technical did not induce increased mutant frequencies in L5178Y $TK^{+/-}$ cells in the presence or absence of S9-mix and is therefore considered non-mutagenic for mammalian cells *in vitro*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate technical

Identification:	Not specified
Description:	White powder
Lot/Batch number:	206-JaK-25-1
Purity:	98.6 %
Stability of test compound:	The stability of the test item at storage conditions (at room temperature in the dark) or in the solvent (vehicle) was not specified.

2. Control material:

Negative control:	Untreated cell cultures which were cultivated in cultivation medium only were included in each experiment.
Solvent (vehicle) control:	As culture medium was used as solvent for the test item, the solvent control represents actually the negative control.
Positive control:	- S9 mix: Ethylnitrosourea (ENU), 100 µg/mL + S9 mix N-nitrosodimethylamine (DMBA), 5 and 10 µg/mL

3. Metabolic activation:

S9 mix was obtained from the livers of Wistar rats weighing approximately 200 g. The animals received a single intraperitoneal injection of Aroclor 1254 at a dose of 500 mg/kg bw. The animals were sacrificed 5 days after treatment following a 16-hour period of fasting until liver homogenates were prepared. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factor.

S9 mix component	Concentration	Unit
HEPES	20	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	3	mM
NADP	4	mM

$MgCl_2$	5	mM
S9	30	% (v/v)

4. Test organism:

L5178Y $TK^{+/-}$ mouse lymphoma cells were used. Stocks were maintained in liquid nitrogen and thawed immediately before use. Each batch used for mutagenicity testing was checked for general morphology, growth characteristics and absence of mycoplasma.

5. Cell culture media:

Cultivation medium: RPMI 1640 medium, supplemented with 10 % horse serum, 200 µg/mL sodium pyruvate and 50 µg/mL gentamycin

Pre-treatment medium A ("THMG medium"): Cultivation medium supplemented with 9 µg/mL hypoxanthine, 15 µg/mL methotrexate and 22.5 µg/mL glycine

Pre-treatment medium B / treatment medium ("THG medium"): Cultivation medium supplemented with 50 % conditioned medium, 9 µg/mL hypoxanthine and 22.5 µg/mL glycine

Selection medium: Cultivation medium, supplemented with 10 % horse serum and 4 µg/mL trifluorothymidine (TFT)

Incubation: *At 37 °C at 5 % CO_2*

6. Locus examined Thymidine kinase (TK)

7. Test concentrations and number of replicates:

(a) Preliminary range-finding assay

Metabolic activation	**Duration of exposure**	**Concentrations**	**Replicates**
-S9 mix	*4 h*	*0.63, 1.3, 2.5 and 5.0 mg/mL*	*Duplicate*
+S9 mix	3 h	0.52, 1.0, 2.1 and 4.2 mg/mL	Duplicate

(b) Main mutation assay:

Metabolic activation	**Duration of exposure**	**Concentrations**	**Replicates**

-S9 mix	*4 h*	*0.63, 1.3, 2.5 and 5.0 mg/mL*		*Duplicate*
+S9 mix	3 h	*0.52, 1.0, 2.1 and 4.2 mg/mL*		Duplicate

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 04 Apr – 13 May 1991
Finalisation date: 10 Sep 1991

2. Preliminary cytotoxicity test:
A preliminary dose-range finding study was performed to determine the concentrations of the test item to be used in the main mutation assay. Pre-treatment of cells and exposure with the test item was conducted under the same conditions as in the main mutagenicity assay.
A series of duplicate cultures was exposed for 4 hours at concentrations in the range of 0.63 – 5.0 mg/mL in the absence of S9 mix and for 3 hours at concentrations in the range of 0.52 – 4.2 mg/mL in the presence of S9 mix. Following treatment, the cultures were diluted and a sample of cells from each culture was seeded (2 cells/well) for the determination of cloning efficiency. In addition, the growth rates of the cultures were monitored for a period of 2 days after treatment. The microtiter plates were incubated for 8-10 days at 37 °C and 5 % CO_2, followed by counting the number of clones and determination of the cloning efficiency.
Based on the results of the preliminary toxicity test, the concentrations for the main mutagenicity assay were chosen. The highest concentration of the main mutagenicity assay was selected to induce 20 % cytotoxicity

3. Main mutation assay:
Pre-treatment of cells:
Thawed cells were maintained at a density of 2×10^5 - 1.5×10^6 in sterile NUNC plastic flasks and incubated at 37 °C and 5 % CO_2. Prior to treatment, spontaneous TK deficient mutants ($TK^{-/+}$ cells) were eliminated from the stock cultures by incubating the cells for one day in THMG medium (pre-treatment medium A), followed by a recovery period of 2-3 days in THG medium (pre-treatment medium B).

Treatment:
In two independent experiments using duplicate cultures per condition, glyphosate was tested at 4 concentrations with and without metabolic activation. Based on the results of the preliminary toxicity test, concentrations for the main mutagenesis assay were selected.

For treatment of cells in the absence of S9 mix, cell suspensions of about 6×10^5 cells/mL were mixed 1:1 with corresponding, 2-fold concentrated test item solutions at concentrations in the range of 0.63 – 5.0 mg/mL prepared in cultivation medium. All cultures were set up in final volumes of 15 mL and incubated under gentle shaking for 4 hours at 37 °C.

For treatment of cells in the presence of S9 mix, pre-treated cell cultures were centrifuged and for each culture 1.8×10^6 cells were re-suspended in 2.5 mL cultivation medium containing the test item at concentrations in the range of 0.52 – 4.2 mg/mL. 0.5 mL S9 mix were added to each culture, followed by incubation for 3 hours under gentle shaking.

In each experiment, negative and positive controls (100 µg/mL ethylnitrosourea in the absence of S9 mix and 5 or 10 µg/mL dimethylbenzanthracene in the presence of S9 mix) were included. At the end of the exposure period, the cells were centrifuged, re-suspended in 15 mL fresh medium and a small sample of cells from each culture was diluted and seeded in a microtiter plate at a density of 2 cells/well for determination of relative cell survival (cloning efficiency 1).

Expression period:
After the exposure period, the cells were incubated for a two days expression period, in which each culture was diluted daily and the growth rate was recorded. After the expression period, each culture was divided. One aliquot of each culture was plated to determine the cell viability (cloning efficiency 2) of the cultures, the other one was used for the selection of mutants.

Selection period:
For the selection of mutants, two microtiter plates were prepared from each post-expression culture, seeding 2000 cells per well in medium supplemented with 4 µg/mL trifluorothymidine (TFT). After an incubation period of 10 days, the number of cell clones was counted. The clones were differentiated into large clones and small dense clones. Small colonies were considered to be associated with clastogenic effects, large colonies were considered to be associated with gene mutation effects.

4. Cytotoxicity:

Cloning efficiency (CE_1 survival)
At the end of the exposure period, a sample of each cell culture was collected to assess cell survival. A full 96-well microtiter plate was seeded at a density of 2 cells/well for each culture. After 10 days of incubation, the number of colonies was counted.

CE_2 (viability)
After the expression period, 2-3 days after end of exposure, a sample of each cell culture was collected to assess cell viability. For each culture, a full 96-well microtiter plate was seeded at a density of 2 cells/well. After 10 days of incubation, the number of colonies was counted.

5. Evaluation:

Cytotoxicity (cloning efficiency CE)
The number of colonies divided by the number of cells plated was calculated for each sample. The absolute cloning efficiency was determined for each test group, as well as the relative cloning efficiency in comparison to the solvent control group.
CE_1 (survival)
The cytotoxicity of the test substance after the exposure period was determined for each test group and is indicated as absolute and relative cloning efficiency (CE_1 and RCE_1, respectively).

CE_2 (viability)
The cytotoxicity of the test substance at the end of the expression period was determined for each test group and is given as absolute and relative cloning efficiency (CE_2 and RCE_2, respectively).
The cloning efficiency (CE, %) was calculated for each test group as follows:

$$\mathrm{CE_x} = -\frac{1}{2}\ln\frac{\text{Number of empty wells}}{\text{Number of wells seeded}}$$

$$\mathrm{RCE_x} = \frac{\mathrm{CE_x}\text{ of the test group}}{\mathrm{CE_x}\text{ of the negative or vehicle control}} \times 100$$

Mutant frequency (MF)
The number of empty wells and the number of wells containing colonies were scored and reported. The colonies are classified into large colonies (indication of gene mutation) and small colonies (indication of chromosome breakage).

Uncorrected mutant frequency:
The uncorrected mutant frequency per 10^4 cells (MF_{uncorr}) was calculated for each test group as follows:

$$\mathrm{MF_{uncorr.}} = -\frac{1}{2000}\ln\frac{\text{Number of empty wells}}{\text{Number of wells seeded}}$$

Corrected mutant frequency
The corrected mutation frequency (MF_{corr}) was calculated regarding the values of CE_2:

$$MF_{corr.} = \frac{MF_{uncorr.}}{CE_2} \times 100$$

Determination of borderline mutant frequency based on GEF
The GEF (global evaluation factor) method requires that the MF exceeds a value based on the global distribution of the background MF of the test method. This value is defined as the mean of the negative/vehicle MF distribution plus one standard deviation.
Based on a large data base (n = 493 experiments) from six laboratories a GEF of 126 mutant colonies per 10^6 cells [mean $MF_{corr} = 99 \times 10^{-6}$ colonies; standard deviation = 27×10^{-6} colonies] was calculated for the microwell method. To be judged positive, the mutation frequency has to exceed a threshold of 126 colonies per 10^6 cells (GEF) above the concurrent negative/vehicle control value. The borderline mutant frequency was calculated for each experiment separately as follows:
Borderline MF = $MF_{vehicle\ control\ corr}$ + GEF (126×10^{-6}).
The borderline MF was not evaluated as part of the present study, but was determined retrospectively for this evaluation.

6. Statistics:
Statistical analysis was conducted using the Analysis of Variance method on the corresponding test and control cultures.

7. Acceptance criteria:
Acceptance criteria were not defined in the study report.

8. Evaluation criteria:
A substance was considered to be mutagenic if the following criteria were met:

- There was a statistically significant and reproducible increase in the mutation frequency as compared to the negative control cultures.
- A dose-response was evident.
- The mutation frequency at the dose level where the highest effect was found more than twice the concurrent spontaneous mutant frequency.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the present study, as not required by the test guideline.

B. CYTOTOXICITY
In the preliminary cytotoxicity test, no cytotoxicity evident as ≥ 20 % reduction of cell survival was observed for any glyphosate concentration, neither in the presence, nor in the absence of metabolic activation. Growth rates of glyphosate treated cells were comparable to those of control cultures (data not provided in study report). Based on these findings, the concentrations used in the preliminary toxicity assay were also applied for the main mutagenicity test.

C. SOLUBILITY
Precipitation of the test item was not reported.

D. MUTANT FREQUENCY
There was no statistically significant increase in the number of mutant colonies observed upon treatment with glyphosate in both experiments at any of the tested concentrations, neither in the presence, nor in the absence of metabolic activation. Mutant frequencies of the medium control cultures were in the expected range.

A clear increase in mutant frequencies was observed for the positive control ethylnitrosourea (100 µg/mL) in the absence of S9 mix. The positive control dimethylbenzanthracene (DMBA) at 5.0 µg/mL showed a rather moderate increase in mutant frequency (1.88 fold induction when compared to medium control), therefore the second experiment was conducted with a higher DMBA concentration of 10 µg/mL. The higher dose revealed a much stronger response in mutant colony formation. Taken together the results of both experiments, the medium and

positive controls demonstrated the sensitivity of the test system and the activity of the S9 mix.

Table B.66.4.1.31-1 : Mutagenicity test: *In vitro* Mammalian Cell Gene Mutation Test with Glyphosate ([redacted] 1991), first experiment

Test group	Mutagenicity data[$]			Toxicity data[$]				Mutagenicity data
	Corrected Mutant Frequency per 10^6 cells			Cloning efficiency (CE_1 -survival)		Cloning efficiency (CE_2 -viability)		Uncorrected mutant frequency Per 10^4 cells
	total	small	large	absolute	relative (RCE_1)	absolute	relative (RCE_2)	Total
Without metabolic activation; 4-hour exposure period								
Medium control	112	50	56	87.0	100.0	78.0	100.0	1.12
MF threshold[§]	238	176	182					
Test item [mg/mL]								
0.61	95	50	42	98.0	112.6	87.0	111.5	0.95
1.30	135	53	76	93.0	106.9	69.0	88.5	1.35
2.50	125	50	69	81.0	93.1	76.5	98.1	1.25
5.00	98	32	62	88.5	101.7	79.5	101.9	0.98
ENU 100 µg/mL	760	120	495	73.5	84.5	71.5	91.7	7.60
IMF	*648*	*71*	*440*					
With metabolic activation; 3-hour exposure period								
Medium control	155	69	77	81.5	100.0	76.0	100.0	1.55
MF threshold[§]	281	195	203					
Test item [mg/mL]								
0.52	145	53	84	90.0	110.4	74.0	97.4	1.45
1.00	145	59	80	88.5	108.6	69.0	90.8	1.45
2.10	130	62	66	93.5	114.7	76.0	100.0	1.30
4.20	160	63	72	78.0	95.7	76.0	100.0	1.60
DMBA 5 µg/mL	510	185	265	46.0	56.4	47.5	62.5	5.10
IMF	*355*	*117*	*188*					

IMF: Induced Mutant Frequency, an increase above vehicle MF, IMF should be $\geq 300 \times 10^{-6}$ for total MF or $\geq 150 \times 10^{-6}$ for small colonies
MF: Mutant frequency; § = $MF_{\text{vehicle control corr}}$ + GEF (126×10^{-6}), rounded
The RMS has added the mutant frequencies for 10^4 cell as reported in the study report.

[$] Mutant frequency values and toxicity data for 10^6 cells. Values differ from those mentioned in study report, where the MF and CE values were given for 10^4 cells.
ENU: Ethylnitrosourea, DMBA: Dimethylbenzanthracene

Table B.6.4.1.31-2: Mutagenicity test: *In vitro* Mammalian Cell Gene Mutation Test with Glyphosate ([redacted]1991), second experiment

Test group	Mutagenicity data[$]			Toxicity data[$]				Mutagenicity data
	Corrected Mutant Frequency per 10^6 cells			Cloning efficiency (CE_1 -survival)		Cloning efficiency (CE_2 -viability)		Uncorrected mutant frequency Per 10^4 cells
	total	small	large	absolute	relative (RCE_1)	absolute	relative (RCE_2)	Total
Without metabolic activation; 4-hour exposure period								
Medium control	225	90	120	64.0	100.0	62.5	100.0	2.25
MF threshold[§]	351	216	246					
Test item [mg/mL]								
0.61	260	113	108	61.5	96.1	52.5	84.0	2.60
1.30	285	109	155	65.0	101.6	55.0	88.0	2.85
2.50	270	107	145	62.5	97.7	50.0	80.0	2.70
5.00	290	104	165	61.5	96.1	53.0	84.8	2.90
ENU 100 µg/mL	1250	245	750	52.5	82.0	43.0	68.8	12.50
IMF	*1025*	*155*	*630*					
With metabolic activation; 3-hour exposure period								
Medium control	330	108	200	61.5	100.0	54.0	100.0	3.30
MF threshold[§]	456	234	326					
Test item [mg/mL]								
0.52	260	91	155	54.5	88.6	56.5	104.6	2.60
1.00	320	123	180	57.5	93.5	49.5	91.7	3.20
2.10	410	140	235	53.5	87.0	52.0	96.3	4.10
4.20	320	110	185	62.0	100.8	60.0	111.1	3.20
DMBA 10 µg/mL	4050	2750	2500	8.0	13.0	3.0	5.6	40.5
IMF	*3720*	*2642*	*2300*					

IMF: Induced Mutant Frequency, an increase above vehicle MF, IMF should be $\geq 300 \times 10^{-6}$ for total MF or $\geq 150 \times 10^{-6}$ for small colonies
MF: Mutant frequency; § = $MF_{\text{vehicle control corr}}$ + GEF (126×10^{-6}), rounded
The RMS has added the mutant frequencies for 10^4 cell as reported in the study report.

[$] Mutant frequency values and toxicity data for 10^6 cells. Values differ from those mentioned in study report, where the MF and CE values were given for 10^4 cells.
ENU: Ethylnitrosourea, DMBA: Dimethylbenzanthracene

III. CONCLUSION:

Based on the experimental findings, glyphosate technical did not induce increased mutant frequencies in L5178Y $TK^{+/-}$ cells in the presence or absence of S9-mix. Under the conditions of the test, the test item is negative for mutagenicity in mammalian cells *in vitro.*

Assessment and conclusion by applicant:

Negative for mutagenicity in L5178Y $TK^{+/-}$ cells with and without metabolic activation.

The study was conducted in compliance with GLP and in accordance with OECD guideline 476 (1983). When compared to the currently valid OECD guideline 490 (2016), a number of deviations became evident, all of them of minor degree and not compromising the validity of the study. Mutant frequency and toxicity data given in the study report included data from 10^4 cells. For this evaluation, data were calculated retrospectively for 10^6 cells. In addition, the borderline mutant frequency based on GEF was determined retrospectively. The study was considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for mutagenicity in L5178Y $TK^{+/-}$ cells in the presence and absence of metabolic activation under the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.32. *In vitro studies – mammalian gene mutation, study 3*

Data point	CA 5.4.1/032
Report author	[redacted]
Report year	1983
Report title	CHO/HGPRT Gene Mutation Assay with Glyphosate
Report No	ML-83-155
Document No	Not reported
Guidelines followed in study	No guideline followed. The study was conducted similarly to OECD 476 (1984)
Deviations from current test guideline OECD 476 (2016)	The newly introduced cytotoxicity parameter RS (relative survival) and the adjusted cloning efficiency were not re-calculated, since no data on the number of cells after treatment were provided. In the current study, cytotoxicity was evaluated based on cloning efficiency after treatment (CE_1, survival) and after selection (CE_2, viability), in accordance with the previous guideline version. The authors did not discriminate between large and small mutant colonies. Historical control data on the medium control and the positive control substances were not included. pH assessments were not performed. The number of cells treated was not reported and the number of cells plated for mutant selection was insufficient. Acceptance and evaluation criteria were not specified. Test results were evaluated inconsistent with the evaluation criteria specified in the current guideline. The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, not concluded under GLP /Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Acceptability/Reliability	Conclusion GRG: Valid, Category 2a Conclusion AGG: The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate (batch: XHJ-64, purity: 98.7 %) was tested for its ability to induce forward mutations in mammalian cells *in vitro* in a HGPRT assay. Chinese hamster ovary (CHO) cells were exposed the test item, medium and positive controls (ethylmethane sulfonate for cultures without S9 mix and benzo(a)pyrene for cultures with S9 mix) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction).

Based on the results of an initial range-finding test (data not provided in study report), in which approximately 90 % cell-killing was observed in the range of 20-25 mg/mL, concentrations for the mutagenicity test were selected. Two independent experiments, one preliminary cytotoxicity and mutagenicity test and one mutation assay were performed in the present study. In the preliminary cytotoxicity and mutagenicity test, glyphosate concentrations in

the range of 5 – 22.5 mg/mL were applied, which were intended to yield approximately 100, 50 and 10 % relative survival of the cells. In addition, S9 mix concentrations in the range of 1-10 % were added to identify the optimal S9 mix concentration. In the main mutagenicity experiment, the cells were exposed to glyphosate concentrations in the range of 2 - 20 mg/mL in the absence of S9 mix and to concentrations in the range of 5 – 25 mg/mL in the presence of S9 mix. Based on the results of the preliminary test, the five test item concentrations for the main mutagenicity assay were chosen to yield 100, 70, 50, 20 and 10 % cell survival at the optimal S9 mix concentration.

After 3 hours of exposure, the cells were incubated for 7-9 days to allow expression of the mutant phenotype. The expression period was followed by a selection period, in which the cells were cultivated in 6-thioguanine-enriched medium for 8-12 days.

Precipitation of the test substance was not reported. In the preliminary cytotoxicity and mutagenicity test, glyphosate-related cytotoxicity was observed at all S9 concentrations tested. In both experiments, cytotoxicity was observed at ≥ 10 mg/mL.

In the preliminary experiment, none of the samples treated with glyphosate exhibited a statistically significant increased mutant frequency compared to the control cultures, neither in the presence nor in the absence of metabolic activation. In addition, mutant frequencies of test item-treated samples were comparable to those of control cultures at all S9 mix concentrations tested. Therefore, an S9 concentration of 5 % was chosen as representative S9 concentration in the main mutagenicity assay.

The negative results were confirmed in the main mutagenicity experiment, in which none of the glyphosate treated samples showed a statistically significant increase in the number of mutant colonies, neither in the presence nor in the absence of metabolic activation. In addition, there was no statistically significant dose-response relationship.

Mutant frequencies of the medium control cultures remained low as expected, whereas the positive control mutagens ethylmethane sulfonate and benzo(a)pyrene yielded large increases in mutant frequencies, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Based on the experimental findings and under the conditions of the test, glyphosate did not induce gene mutations in the HGPRT locus, neither in the presence nor in the absence of metabolic activation and is therefore considered negative for mutagenicity in mammalian cells *in vitro*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	Not specified
Description:	White powder
Lot/Batch number:	XHJ-64
Purity:	98.7 %
Stability of test compound:	The stability of the test item at storage conditions (at room temperature) or in the solvent (vehicle) was not specified.

2. Control material:

Negative control:	Untreated cell cultures which were cultivated in cultivation medium only were included in each experiment.
Solvent (vehicle) control:	As culture medium was used as solvent for the test item, the solvent control represents actually the negative control.
Positive control:	- S9 mix: Ethyl methane sulphonate (EMS), 200 µg/mL + S9 mix Benzo(a)pyrene (B(a)P), 2 µg/mL

3. Metabolic activation:

S9 mix was purchased from [redacted]. The liver homogenate was produced from Aroclor 1254-induced rats and prepared before the experiment by mixing S9 fraction and co-factor as follows:

S9 mix component	Concentration	Unit
Sodium phosphate buffer (pH 7.5)	50	mM
KCl	30	mM
NADPH-generating system		

Glucose 6-phosphate	5	mM
NADP	4	mM
$MgCl_2$	10	mg
$CaCl_2$	10	mM
S9*	5	% (v/v)

* In a preliminary experiment, the optimal S9 concentration was determined; 1, 2, 5 and 10 % (v/v) of S9 mix were added to the co-factor solutions. 5 % (v/v) of S9 fraction in the co-factor mix were used for the main mutagenicity study.

4. Test organism:

Chinese hamster ovary (CHO) cells (K1BH4) were used. The cells were routinely maintained as logarithmically growing monolayer cultures in Ham's F12 medium.

5. Cell culture media:

Cultivation medium: Ham's F12 medium, supplemented with 10 % newborn calf serum

Growth medium: Ham's F12 medium, supplemented with 10 % dialysed newborn calf serum

Treatment medium (± S9): *Ham's F12 medium*

Selection medium: Ham's F12 medium supplemented with 5 % dialysed newborn calf serum and 10 µM 6-thioguanine (6TG)

Incubation: *At 37.5 ± 2 °C, 5 % CO_2 and 95 % humidity*

6. Locus examined: Hypoxanthin guanine phosphoribosyltransferase (HGPRT)

7. Test concentrations and number of replicates:

(a) Preliminary cytotoxicity and mutagenicity test:

Metabolic activation	Duration of exposure	Concentrations	Replicates
±S9 mix	*3 h*	*5, 17.5 and 22.5 mg/mL*	*Duplicate*

(b) Main gene mutation test:

Metabolic activation	Duration of exposure	Concentrations	Replicates
-S9 mix	*3 h*	*2, 5, 10, 15 and 20 mg/mL*	*Duplicate*
+S9 mix	3 h	*5, 10, 15, 20 and 25 mg/mL*	Duplicate

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 21 Jun – 09 Sep 1983

Finalisation date: 20 Oct 1983

2. Preliminary cytotoxicity and mutagenicity test:

A preliminary test was performed to initially estimate the mutagenic potential of the test item and to identify the optimal S9 mix concentration.

Based on the results of an initial range-finding test (data not provided in study report), in which approximately 90 % cell-killing was observed in the range of 20 - 25 mg/mL, glyphosate concentrations in the range of 5 – 25 mg/mL were applied in the present preliminary cytotoxicity and mutagenicity test. The test item concentrations for the preliminary test were selected to yield approximately 100, 50 and 10 % relative survival of the cells.

The procedure of the preliminary test was the same as for the main mutation assay described below. The cells were treated under the same conditions as in the main mutagenicity assay at concentrations of 5, 17.5 and 22.5 mg/mL in the presence and absence of metabolic activation. To identify the optimal S9 mix concentration, 1, 2, 5 or 10 % of S9 mix were added to cultures with metabolic activation. Each two cultures per condition were exposed for 3 hours with and without S9 mix. Following exposure, cells were plated for determination of cell survival (cloning efficiency 1) and mutagenicity. Mutant cells were allowed for expression of the mutant phenotype for 7 - 9 days, followed by an 8 - 12 day selection period in 6-thioguanine-enriched medium. In addition, the cell viability (cloning efficiency 2) was determined for mutant cultures in the presence and absence of selection medium. Mutagenicity was assessed by calculation of the mutant frequency for all tested conditions.

Based on the results of the preliminary test, the five test item concentrations for the main mutagenicity assay were chosen to yield 100, 70, 50, 20 and 10 % cell survival at the optimal S9 mix concentration.

3. Main mutation assay:

Pre-treatment of cells:

For each test group, 0.5 x 10^6 cells per flask were seeded into 25 cm^2 flasks and incubated for 18 - 24 hours prior to treatment.

Treatment:

On the day of treatment, the medium was changed to serum-free treatment medium and, for treatment with metabolic activation, 5 % S9 mix was added. The cells were exposed to glyphosate concentrations in the range of 2 - 20 mg/mL in the absence of S9 mix and to concentrations in the range of 5 – 25 mg/mL in the presence of S9 mix for 3 hours at 37.5 ± 2 °C. Solvent (medium) and the positive controls (ethylmethane sulfonate for cultures without S9 mix and benzo(a)pyrene for cultures with S9 mix) were included. After exposure, the cells were washed with Hank's Buffered Salt Solution (HBSS), trypsinised and counted. 200 cells per sample were seeded for the determination of survival (cloning efficiency 1) after the exposure period and 10^6 cells per sample were seeded to assess mutagenicity.

Expression period:

After treatment, 10^6 cells per culture were plated in 10 mL growth medium and incubated for an expression period of 7 - 9 days. The cells were sub-cultured every 2 - 3 days to maintain exponential growth during the expression time. After the expression period, each culture was divided. One aliquot of each culture was plated to determine the viability (cloning efficiency 2) of the cultures in the absence of selection medium, the other one was used for the selection of mutants.

Selection period:

For the selection of mutants, 10^6 cells of each post-expression culture were seeded into 100 mm plates (5 plates with 2 x 10^5 cells per culture) containing 8 mL of 6-thioguanine enriched selection medium. After an incubation period of 8 - 12 days, the developed colonies were fixed, stained and counted.

4. Cytotoxicity:

Cloning efficiency CE_1 (survival)

The survival (cloning efficiency 1) of glyphosate treated cells relative to solvent controls was determined in parallel to the mutagenicity test. At the end of the exposure period, a sample of each cell culture was collected to assess survival of the cells. 200 cells per culture were plated and re-incubated for 7 - 9 days. After the incubation period, the colonies developed were fixed, stained and counted.

Cloning efficiency CE_2 (viability)

The viability (cloning efficiency 2) was determined in parallel to the selection of mutants. After the expression period, 200 cells of each culture were plated in triplicates in selection medium without 6-thioguanine to assess cell viability. After an incubation period of 8 - 12 days, the developed colonies were fixed, stained and counted.

5. Evaluation:

Cytotoxicity (cloning efficiency CE)
The number of colonies divided by the number of cells plated was calculated for each sample. The absolute cloning efficiency was determined for each test group, as well as the relative cloning efficiency in comparison to the solvent control group.

CE_1 (survival)
The cytotoxicity of the test substance after the exposure period was determined for each test group and is indicated as absolute and relative cloning efficiency (CE_1 and RCE_1, respectively).

CE_2 (viability)
The cytotoxicity of the test substance at the end of the expression period was determined for each test group and is given as absolute and relative cloning efficiency (CE_2 and RCE_2, respectively).

The cloning efficiency (CE, %) was calculated for each test group as follows:

$$\mathrm{CE_{absolute}} = \frac{\text{Total number of colonies}}{\text{Total number of cells plated}} \times 100$$

$$\mathrm{RCE_x} = \frac{\mathrm{CE_{absolute}}\ \text{of the test group}}{\mathrm{CE_{absolute}}\ \text{of the vehicle control group}} \times 100$$

Mutant frequency (MF)
The cloning efficiency of mutant colonies in selective medium divided by the cloning efficiency in non-selective medium measured for the same culture at the time of selection was calculated for each sample.

Uncorrected mutant frequency:
The uncorrected mutant frequency (MF_{uncorr}) was calculated for each test group as follows:

$$\text{MF uncorrected} = \frac{\text{Total number of mutant colonies}}{\text{Number of seeded cells}} \times 10^6$$

Corrected mutant frequency
The corrected mutation frequency (MF_{corr}) was calculated regarding the values of CE_2:

$$\text{MF corrected} = \frac{\text{MF uncorrected}}{\mathrm{CE_2}} \times 100$$

6. Statistics:
Mutagenicity data were analysed according to the statistical method of Snee and Irr (1981). Mutant frequency values were transformed according to the equation $Y = (X+1)^{0\,15}$, with Y = transformed mutant frequency and X = observed mutant frequency. Student's t-test was then used to compare treatment data to solvent control data.

7. Acceptance criteria:
Acceptance criteria were not specified in the study report.

8. Evaluation criteria:
Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS
Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

In the preliminary cytotoxicity and mutagenicity test, glyphosate-related cytotoxicity was observed at all S9 concentrations tested. In both the preliminary and in the main mutagenicity experiment, the test material was shown to be cytotoxic at concentrations of 10 mg/mL and above.

C. SOLUBILITY

Information on precipitation or pH changes of glyphosate in the medium have not been provided in the study report.

D. MUTANT FREQUENCY

In the preliminary experiment, none of the samples treated with glyphosate exhibited a statistically significantly increased mutant frequency compared to the control cultures, neither in the presence nor in the absence of metabolic activation. In addition, mutant frequencies of test item-treated samples were comparable to those of control cultures at all S9 mix concentrations tested. Therefore, an S9 concentration of 5% was chosen as representative S9 concentration in the main mutagenicity assay.

In the main mutagenicity experiment, there was as well no statistically significant increase in the number of mutant colonies upon treatment with glyphosate, neither in the presence nor in the absence of metabolic activation. In addition, there was no statistically significant dose-response relationship.

Mutant frequencies of the medium control cultures remained low as expected, whereas the positive control mutagens ethylmethane sulfonate and benzo(a)pyrene yielded large increases in mutant frequencies, demonstrating the sensitivity of the test and the functionality of the S9 mix.

Table B.64.1.32-1: CHO/HGPRT Gene Mutation Assay with Glyphosate ([redacted], 1983), preliminary test

Test group	Mutant frequency (per 10^6 cells)		Cloning efficiency			
			CE_1 (survival, %)		CE_2 (viability, %)	
	uncorr.	corr.[#]	abs.	rel.	abs.	rel.
Without metabolic activation; 3-hour exposure period						
Medium control	5.00	7.40	63.50	100.00	69.92	100.00
Test item [mg/mL]						
5.0	5.00	7.10	70.58	111.15	71.58	102.38
17.5	9.50	15.40	42.00	66.14	61.67	88.20
22.5	3.00	5.30	6.60	10.39	59.83	85.58
Positive control [µg/mL]						
(EMS) 200.0	85.50	**163.70**	58.20	91.65	52.42	74.97
With metabolic activation (5% S9); 3-hour exposure period						
Medium control	4.50	4.40	78.25	100.00	106.08	100.00
Test item [mg/mL]						
5.0	7.50	6.40	84.58	108.09	117.00	110.29
17.5	4.00	6.90	65.33	83.49	52.67	49.65
22.5	5.50	8.50	51.83	66.24	65.83	62.06
Positive control [µg/mL]						
(B(a)P) 2.0	73.00	**121.20**	36.25	46.33	60.92	57.42

[#] Correction on the basis of the absolute cloning efficiency 2 at the end of the expression period

Data shown for samples with 5 % S9 mix only

Values were re-calculated based on raw data given in the study report

Table B.6.4.1.32-2: CHO/HGPRT Gene Mutation Assay with Glyphosate [redacted], 1983), main mutagenicity test

Test group	Mutant frequency (per 10^6 cells)		Cloning efficiency			
			CE_1 (survival, %)		CE_2 (viability, %)	
	uncorr.	corr.[#]	abs.	rel.	abs.	rel.
Without metabolic activation; 3-hour exposure period						
Medium control	8.30	11.30	51.06	100.00	74.94	100.00
Test item [mg/mL]						
2.0	2.30	3.50	50.39	98.69	67.61	90.21
5.0	7.30	11.30	47.39	92.82	66.83	89.18
10.0	8.30	10.80	46.06	90.21	74.44	99.33
15.0	14.70	20.80	52.89	103.59	68.56	91.48
20.0	7.00	10.10	19.56	38.30	69.17	92.29
Positive control [µg/mL]						
(EMS) 200.0	83.00	**135.40**	46.94	91.95	61.28	81.76
With metabolic activation (5% S9); 3-hour exposure period						
Medium control	5.70	7.70	49.22	100.00	75.72	100.00
Test item [mg/mL]						
5.0	4.00	5.70	55.64	113.04	69.75	92.11
10.0	9.30	13.10	49.29	100.13	68.56	90.54
15.0	6.00	9.90	55.44	112.64	60.13	79.40
20.0	9.00	14.90	47.50	96.50	56.86	75.09
25.0	9.30	13.10	22.11	44.92	68.44	90.39
Positive control [µg/mL]						
(B(a)P) 2.0	17.70	**76.80**	23.17	47.07	67.22	88.77

[#] Correction on the basis of the absolute cloning efficiency 2 at the end of the expression period

Values were re-calculated based on raw data given in the study report

III. CONCLUSION:

Based on the experimental findings and under the conditions of the test, glyphosate did not induce gene mutations in the HGPRT locus, neither in the presence nor in the absence of metabolic activation and is therefore considered negative for mutagenicity in mammalian cells *in vitro*.

Assessment and conclusion by applicant:

Negative for mutagenicity at the HGPRT locus in CHO cells with and without metabolic activation.
This non-GLP study was performed equivalent to OECD 476 (2016). Although a number of deviations became evident when compared with the currently valid guideline, these were considered to be of minor degree and to not compromise the validity of the study. Therefore, the study is considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for mutagenicity at the HGPRT locus in CHO cells in the presence and absence of metabolic activation under

the conditions of this test. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.1.33. *In vitro studies – UDS assay, study 1*

Data point	CA 5.4.1/033
Report author	[redacted]
Report year	1994
Report title	DNA repair test with primary rat hepatocytes
Report No	931564
Document No	Not reported
Guidelines followed in study	OECD 482 (1986)
Deviations from current test guideline	Not applicable. OECD 482 was deleted in 2014. When compared to the previous OECD 482 (1986), several deviations became evident. Instead of autoradiography or liquid scintillation counting procedures, incorporation of radioactivity into the DNA was determined based on UV absorbance and mathematical calculations. According to OECD 482 (1986) at least two cell cultures per condition are required. In the current study only one culture per condition was tested. However, a second experiment at concentrations in a similar range was conducted. As no individual value of glyphosate treated cultures at all showed an increase in ^{3}H-dCyd, this deviation seems to not compromise the validity of the study.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Conclusion GRG: supportive, Category 2a Conclusion AGG: The study is considered to be not acceptable due to the noted deviations and since the UDS assay is no longer a standard method.

Glyphosate (batch: F/93/032, purity: > 98 %) was tested in primary rat hepatocytes *in vitro* for its ability to induce unscheduled DNA synthesis (UDS) according to the bromodeoxyuridine density shift method. Hepatocytes were isolated from the livers of adult male Sprague-Dawley rats and used for two separate experiments. Based on the results of a preliminary solubility and cytotoxicity test (data not provided in the study report), appropriate glyphosate concentrations were selected for the UDS assay.

In both experiments, freshly isolated hepatocytes were exposed to test item concentrations in the range of 0.20 – 48.98 mM (first experiment) and 1.14 – 111.69 mM (second experiment) in the presence of tritiated deoxycytidine (^{3}H-dCyd) and bromodeoxyuridine (BrdUrd). Medium and positive controls (dimethylnitrosamine (DNM) and 2-acetamidofluorene (2-AAF)) were included in each experiment. After 18 hours of incubation, the cells were lysed, nucleated DNA was extracted and the replicated, BrdUrd containing DNA as well as the repaired ^{3}H-dCyd and BrdUrd containing DNA were separated by centrifugation on an alkaline cesium salt gradient. Repaired and replicated DNA was quantified by measuring the incorporated radioactivity. Genotoxicity was evaluated based on the induction of DNA repair and expressed as incorporated ^{3}H-dCyd per µg DNA, whereas cytotoxicity was assessed based on the degree of inhibition of replicative DNA synthesis (incorporated BrdUrd per µgDNA).

Precipitation of the test substance in medium, as well as cytotoxic effects were observed at 111.69 mM in the second experiment only. Treatment with glyphosate did not induce a significant increase in DNA repair above those of control conditions in none of the experiments at any tested concentration. Incorporation of ^{3}H-dCyd of the medium control was within the normal range of control data, while the two positive controls DNM and 2-AAF showed a significant increase in ^{3}H-dCyd incorporation, confirming the responsiveness and metabolic activity of the test system.

Under the conditions of the test, glyphosate did not induce unscheduled DNA synthesis in primary rat hepatocytes *in vitro*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	Not specified
Description:	Not specified
Lot/Batch number:	F/93/032
Purity:	> 98 %
Stability of test compound:	The stability of the test item at storage conditions (light protected, at 20 – 25 °C) or in solvent was not specified.

2. Control material:

Negative control:	Untreated cell cultures which were cultivated in cultivation medium only were included in each experiment.
Solvent (vehicle) control:	As culture medium was used as solvent for the test item, the solvent control represents actually the negative control.
Positive controls:	Dimethylnitrosamine (DMN, 10 mM) and 2-acetamidofluorene (2-AAF, 0.2 mM)

3. Hepatocyte isolation:

Primary rat hepatocytes were obtained by *in situ* collagenase perfusion from the livers of a single adult male Sprague-Dawley rat weighing approximately 200 – 350 g. The liver was perfused with a Ca^{2+}-free modified Hank's solution containing 100 µM EGTA, continued by the same Ca^{2+}-free solution without EGTA. Finally, the liver was perfused with Dulbecco's modified Eagle's medium (DMEM) including Ca^{2+} and collagenase (0.13 U/mL) and harvested. After filtration through a nylon mesh gauze, the cells were washed in DMEM three times and the viability was determined by trypan blue exclusion. Only hepatocyte preparations with a viability > 80 % were used.

4. Cell culture:

Cell culture establishment:	Freshly isolated hepatocytes were seeded in plating medium at a density of 6 x 10^6 cells in 75 cm^2 flasks, incubated and allowed to attach for 2 hours. Afterwards, the cells were washed twice with pre-warmed salt solution prior to treatment.
Plating medium:	William's medium E, supplemented with 10 % fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin
Pre-treatment medium:	*William's medium E, supplemented with 40 µM fluorodeoxyuridine (FdUrD) and 200 µM Bromodeoxyuridine (BrdUrd)*
Treatment medium:	*William's medium E, supplemented with 40 µM FdUrD, 200 µM BrdUrd and 10 µCi/mL ^{3}H-deoxycytidine (^{3}H-dCyd)*
Incubation:	*Not specified*

5. Test concentrations:

Experiment	Concentrations
First experiment:	0.20, 0.61, 1.81, 5.44, 16.32 and 48.98 mM
Second experiment:	1.14, 3.41, 10.23, 30.69, 92.08 and 111.69 mM

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 01 Feb – 18 Mar 1994
Finalisation date: 28 Mar 1994

2. Cytotoxicity

A preliminary dose-range finding test was not conducted as part of the described study. However, solubility and cytotoxicity have been examined in a pre-test (data not provided in study report), which enabled the selection of the test item concentrations for the UDS assay. In the present study, cytotoxicity was estimated based on the degree of inhibition of replicative DNA synthesis.
The degree of cytotoxicity was therefore determined as part of the genotoxicity test (described below), indicated by a reduction in the incorporation of radioactivity in semiconservatively replicated (dense) DNA strands.

3. Unscheduled DNA synthesis

Cell treatment and harvest:
After cell culture establishment, the cells were incubated in pre-treatment medium containing fluorodeoxyuridine(FdUrD) and BrdUrd for one hour. Following pre-incubation, the medium was exchanged to treatment medium containing additionally ^{3}H-dCyd.

Two independent experiments were performed in parallel, using hepatocytes from the same animal. The cells were exposed to test item concentrations in the range of 0.20 – 48.98 mM (first experiment) and 1.14 - 111.69 mM (second experiment). Medium and positive control cultures (10 mM dimethylnitrosamine and 0.2 mM 2-acetamidofluorene) were included in each experiment.

After 18 hours of incubation, the cells were washed with phosphate buffered saline, lysed with sodium dodecylsarcosine and digested with proteinase K.

Preparation of DNA and determination of radioactivity:
The DNA was precipitated with ethanol, dried and stored at -80 °C. DNA pellets were dissolved and centrifuged to equilibrium in alkaline $CsCl/Cs_2So_4$ gradients at 20 °C for 16 hours. Following centrifugation, the lower half of each gradient was removed. The remaining part containing parental DNA strands was mixed with $CsCl/Cs_2SO_4$ rebanded by centrifugation. After fractionation of the gradients, the UV absorbance and the acid precipitable incorporated radioactivity were determined for each fraction.

Evaluation:
Repair synthesis (cpm/µg DNA) was estimated from the gradient profiles by calculating the radioactivity incorporated into light (parental) DNA, i. e. by integrating the radioactivity binding exactly coincident with the UV-absorbance peak, and dividing it by the amount of parental DNA. DNA was quantitated by integrating the UV-absorbance peak and converting it into μg DNA by means of a calibration curve. Normal (semiconservative) DNA synthesis (cpm/µg DNA) was determined from the gradient profiles as radioactivity incorporated into dense DNA.

4. Statistics:

Statistical analysis was not performed in the study.

5. Acceptance criteria:

Acceptance criteria were not defined in the study report.

6. Evaluation criteria:

A substance was considered genotoxic if it produced a reproducible and significant dose-related increase in radiolabel incorporation. A substance was considered non-genotoxic if it produced neither a significant, dose-related increase in ^{3}H incorporation, nor a reproducible positive response at any test point.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. CYTOTOXICITY

Cytotoxicity, indicated by inhibition of replicative DNA synthesis, was observed in the second experiment only at a glyphosate concentration of 111.69 mM (refer to Table B.6.4.1.33-1).

C. SOLUBILITY

Precipitation of the test item in culture medium was observed at the highest concentration of 111.69 mM.

D. UNSCHEDULED DNA SYNTHESIS

There was no significant increase in ^{3}H-dCyd incorporation for glyphosate treated hepatocytes when compared to control levels observed, in any experiment and at any tested concentration.

Radiolabel incorporations of ^{3}H-dCyd in the untreated medium control cultures were within the variability of the control values of individual experiments. The two positive compounds DMN and 2-AAF markedly enhanced DNA repair in the hepatocytes, confirming the responsiveness and metabolic activity of the test system.

Table B.6.4.1.33-1: DNA repair test with primary rat hepatocytes (UDS assay) ([redacted] 1994)

	Genotoxicity	Cytotoxicity
	Repair synthesis [cpm/µg DNA]	**Replicative synthesis [cpm/µg DNA]**
First experiment		
Medium control[#]	112.7	2442.2
Test item [mM]		
0.20	114.8	1998.4
0.61	109.4	2363.0
1.81	115.1	2641.0
5.44	102.4	2509.1
16.32	109.9	2356.6
48.98	98.2	1729.1
Positive controls		
DMN [10 mM]	973.7	1794.9
2-AAF [0.2 mM]	817.4	306.2
Second experiment		
Medium control[#]	95.5	2573.7
Test item [mM]		
1.14	96.2	2784.2
3.41	88.5	2514.7
10.23	95.7	2902.6
30.69	87.8	3175.0
92.08	71.6	2664.6
111.63	66.9	737.2
Positive controls		
DMN [10 mM]	928.5	1521.8

Table B.6.4.1.33-1: DNA repair test with primary rat hepatocytes (UDS assay) ([redacted] 1994)

	Genotoxicity	Cytotoxicity
	Repair synthesis [cpm/µg DNA]	**Replicative synthesis [cpm/µg DNA]**
2-AAF [0.2 mM]	728.5	829.9

2-AAF: 2-acetamidofluorene; DMN: Dimethylnitrosamine
[#] Mean value of 2 replicates, calculated based on raw data given in study report

III. CONCLUSION:

Under the conditions of the test, glyphosate did not induce any increase in tritiated cytidine incorporation more than 10 % when compared to control values. Thus, glyphosate did not induce DNA damage leading to unscheduled DNA synthesis in primary rat hepatocytes *in vitro*.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for unscheduled DNA synthesis in primary rat hepatocytes *in vitro*.

The study was performed under GLP conditions and in accordance with OECD guideline 482 (1986), which was deleted in 2014. As the Unscheduled DNA Synthesis (UDS) assay is no longer a standard method described by current guidelines, the study was considered to provide supporting information. When compared to OECD guideline 482 (1986), a number of deviations became evident, all of them of minor degree and not compromising the validity of the study.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for unscheduled DNA synthesis in primary rat hepatocytes *in vitro* under the conditions of this test. Considering that the UDS assay is no longer a standard method, the study was considered not acceptable though.

B.6.4.1.34. *In vitro studies - UDS assay, study 2*

Data point:	CA 5.4.1/034
Report author	[redacted]
Report year	1983
Report title	The hepatocyte primary culture / DNA repair assay on compound JJN-1020 using rat hepatocytes in culture
Report No	AH-83-181
Document No	M-645649-01-1
Guidelines followed in study	Similar to OECD 482 (1986)
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate (batch: XHJ-64, purity not specified in study report, 98.7% reported in other study reports and doc J of applicant) was tested for unscheduled DNA synthesis (UDS) in primary rat hepatocytes *in vitro*. Hepatocytes were isolated from the livers of adult male F344 rats and triplicate cultures per condition were exposed to test item concentrations in the range of 0.0125 to 125 µg/mL in medium supplemented with radiolabeled tritiated thymidine (^{3}H-TdR). Medium, solvent (DMSO and 0.1 N NaOH), negative (50 µM pyrene) and positive controls (50 µM benzo(a)pyrene) were tested in parallel. After 18 – 20 hours of incubation at 37 °C, the cells were processed for

	slide preparation and autoradiographs developed. Cytotoxicity was assessed by the absence of S-phase cells in the autoradiographs and by morphology. Unscheduled DNA synthesis was quantified by determining the net increase in nuclear grain counts induced by the test item or corresponding controls in a total of 20 - 80 cells per culture.
Short description of results:	Precipitation of the test item in culture medium was not reported and there was no cytotoxicity observed up to the highest tested concentration of 125 µg/mL. Glyphosate did not induce a significant increase in the mean number of net nuclear grain counts when compared to solvent controls at any of the tested concentrations. The values obtained with the solvent and negative controls were in the expected range, whereas the positive control B(a)P significantly increased the number of mean net nuclear grain counts, thus demonstrating the sensitivity of the test system towards DNA damaging substances. Based on the experimental findings and under the conditions of the test, glyphosate did not induce unscheduled DNA synthesis indicated by DNA repair activity in primary rat hepatocytes *in vitro*.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	The study was not conducted under GLP. The corresponding OECD 482 (1986) was deleted in 2014. When compared to OECD 482 (1986), a number of deviations became evident. The selection of test concentrations was not justified in the study report and the highest concentration tested did not cause cytotoxicity. According to OECD 482 (1986) at least 50 cells per culture should be counted and evaluated for nuclear grain counts. In the present study, 20 -80 cells per culture were evaluated. A confirmatory experiment was performed, but raw data of the repeat assay were not included in the study report. The study is therefore considered not acceptable. **Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since when compared to OECD 482 (1986), a number of deviations became evident. Besides, the UDS assay is no longer a standard method.

B.6.4.1.35. *In vitro studies – Rec assay, study 1*

Data point	CA 5.4.1/035
Report author	[redacted]
Report year	1995
Report title	HR-001: DNA Repair Test (Rec-Assay)
Report No	IET 94-0141
Document No	Not reported
Guidelines followed in study	U.S. EPA FIFRA Guidelines, Subdivision F
Deviations from current test guideline	Not applicable. The Rec-assay is not a standard method for this endpoint (DNA damage and repair). Furthermore, the dose selection was not explained and viability data were not included in the study report.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Conclusion GRG: supportive, Category 2a Conclusion AGG: The study is considered to be supportive due to the noted deviations. Rec assay is not a standard method for DNA damage and repair.

The DNA-damaging activity of glyphosate (HR-001, batch: 940908-1, purity: 95.68 %) was investigated in an DNA repair test with *Bacillus subtilis* strains H17 and M45. In a well diffusion assay, the bacteria were exposed to test item concentrations in the range of 7.5 to 240 µg/disk in the presence and absence of metabolic activation (phenobarbital and 5,6 benzoflavone-induced rat liver S9 fraction). Each concentration was tested in duplicates.

Vehicle (sterile water), negative (kanamycin) and positive controls (mitomycin c without S9 mix and 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indole (Trp-p-1) in the presence of S9 mix) were included. After an incubation time of 24 h at 37 °C the diameter of the growth inhibition zone was measured for each strain.

Precipitation of the test item was not reported.

A relevant growth inhibition of bacteria strains H17 (rec^+) or M45 ($recE^-$) was not observed at any tested condition, neither in the presence, nor in the absence of metabolic activation. Although a growth inhibition zone of 1 mm diameter was noted at the highest test concentration of 240 µg/disk in strain M45 in the absence of metabolic activation, the differences of growth inhibitory zones between the strains H17 and M45 were 1 mm or less. There was no growth inhibition in strain M45 in the presence of S9 mix nor in strain H17 in the presence or absence of S9 mix.

Positive, negative and vehicle controls showed the expected results and demonstrated the validity of the test system and the functionality of the S9 mix.

Based on the results of the present study, glyphosate technical has no DNA-damaging activity in bacteria.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate

Identification:	HR-001
Description:	Solid crystals
Lot/Batch #:	940908-1
Purity:	95.68 %
Stability of test compound:	The stability of the test item at storage conditions (in a dark cold room at approx. 5 °C) or in the solvent (vehicle) were not specified.
Solvent (vehicle) used:	Sterile water

2. Control materials:

Negative control:	Kanamycin, 0.2 µg/disk
Solvent (vehicle) control:	Sterile water
Solvent (vehicle) /final concentration:	20 µL/disk
Positive controls:	- S9 mix: Mitomycin C, 0.01 µg/disk + S9 mix: 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indole (Trp-p-1), 5 µg/disk

3. Metabolic activation:

S9 mix was purchased from [redacted] Lot no. RAA-314). The homogenate was produced from the livers of 7 weeks old male Sprague-Dawley rats, weighing 188 – 238 g, that received intraperitoneal injections of phenobarbital (30 mg/kg bw on Day 1, each 60 mg/kg bw on Days 2, 3 and 4) and 80 mg/kg bw 5,6 benzoflavone on Day 3. The S9 mix was prepared immediately before the experiment by mixing S9 fraction and co-factor.

S9 mix component	**Concentration**	**Unit**
Sodium phosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADH	4	mM
NADP	4	mM
$MgCl_2$	8	mM
S9	1	% (v/v)

4. Test organisms:

Tester strains			
B. subtilis		**Bacteria batch checked for**	
Recombination wild (rec^+) H17	✓	UV-light sensitivity (recE)	✓
Recombination deficient ($recE^-$) M45	✓	Response to negative and positive control chemicals	✓

5. Test concentrations:

Diffusion assay ± S9 mix:	
Concentrations:	*7.5, 15, 30, 60, 120 and 240 µg/disk*
Tester strains:	H17, M45
Replicates:	*Duplicates in a single experiment*

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 14 – 15 Feb 1995
Finalisation date: 14 Mar 1995

2. Diffusion assay
0.1 mL pre-cultured bacterial suspension (3 x 10^7 cells/mL), 5 mL molten B2 top agar and, in experiments with metabolic activation, 0.05 mL of S9 mix were mixed uniformly in an 90 mm petri dish and the agar plates were left at room temperature for solidification. Paper discs (8 mm diameter) impregnated with 20 µL test solution, positive, negative or vehicle control were placed on the prepared spore agar plate. Each concentration was tested in duplicates. After an incubation time of 24 h at 37 °C the diameter of the growth inhibition zone was measured for each strain.

3. Statistics
Results were judged without statistical analysis.

4. Acceptance criteria
The test was valid if

- Growth inhibition was not observed in solvent controls of either strain.
- For the positive control, the growth inhibitory zone in strain M45 was larger than the zone in strain H17 and the difference in diameter was ≥ 5 mm.
- For the negative control, the difference in diameter of growth inhibitory zone between the strains was ≤ 4 mm.

5. Evaluation criteria
In case the test item caused growth inhibition in at least one strain, results were judged positive when the following criteria were met:

- The growth inhibitory zone of M45 was larger than that of H17 and
- The difference in diameter was 5 mm or more at one or more dose levels that caused growth inhibitory zones with diameters of 4 mm or less in the H17 strain.

If a positive result was obtained, a re-test was conducted to confirm the reproducibility of the positive result.

Results were judged negative when the test substance causes no growth inhibition in either strain.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required for this type of *in vitro* study.

B. SOLUBILITY

Precipitation of the test substance was not reported.

C. MUTATION ASSAY

A relevant growth inhibition of bacteria strains H17 (rec^+) or M45 ($recE^-$) was not observed at any tested condition, neither in the presence, nor in the absence of metabolic activation. Although a growth inhibition zone of 1 mm diameter was noted at the highest test concentration of 240 µg/disk in strain M45 in the absence of metabolic activation, the differences of growth inhibitory zones between the strains H17 and M45 were 1 mm or less. There was no growth inhibition in strain M45 in the presence of S9 mix or in strain H17 in the presence nor absence of S9 mix.

Negative controls treated with kanamycin induced growth inhibition zones with differences between 2 - 3 mm between M45 and H17, while positive controls treated with mitomycin C (-S9 mix) or Trp-p-1 (+S9 mix) caused large growth inhibitory zones with differences in diameter of 19 and 11 - 12 mm.

Table B.6.4.1.35-1: DNA Repair Test (Rec-Assay) ([redacted] 1995)

Compound	Dose (µg/disk)	S9 fraction (-)			S9 fraction (+)		
		Inhibitory zone** (mm)		Difference*** (mm)	Inhibitory zone** (mm)		Difference*** (mm)
		M45	H17		M45	H17	
Solvent control (H_2O)		0	0	0	0	0	0
		0	0	0	0	0	0
Test item	7.5	0	0	0	0	0	0
		0	0	0	0	0	0
	15	0	0	0	0	0	0
		0	0	0	0	0	0
	30	0	0	0	0	0	0
		0	0	0	0	0	0
	60	0	0	0	0	0	0
		0	0	0	0	0	0
	120	0	0	0	0	0	0
		0	0	0	0	0	0
	240	1	0	1	0	0	0
		0	0	0	0	0	0
Negative control (Kanamycin)	0.2	8	6	2			
		9	6	3			

Positive control (Mitomycin C)	0.01	20	1	19			
		20	1	19			
Positive control (Trp-p-1)*	5				11	0	11
					12	0	12

*Trp-p-1: 3-amino-1,4-dimethyl-5H-pyrido [4,3-b] indole, positive control in the presence of S9 mix
**Diameter of growth inhibitory zone substracted the diameter of disk.
***Diameter of growth inhibitory zone in M45 strain substracted that in H17 strain..

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III. CONCLUSION

According to the results of the present study and under the experimental conditions chosen, the test item does not show DNA-damaging activity in the DNA repair test (Rec-Assay) in the presence or absence of metabolic activation.

3. Assessment and conclusion

Assessment and conclusion by applicant:
Negative for genotoxicity in bacteria (*B. subtilis* H17 and M45) with and without metabolic activation.
The study was conducted under GLP conditions and according to US EPA FIFRA guidelines subdivision F. It was considered to provide supplementary information only because the Rec assay is not a standard method for this endpoint (DNA damage and repair). Furthermore, the dose selection was not explained and viability data were not included in the study report.

Assessment and conclusion by RMS: : It is agreed with the applicant's conclusion. Glyphosate was negative for genotoxicity in bacteria (*B. subtilis* H17 and M45) in the presence and absence of metabolic activation under the conditions of this test. The study is considered supportive due to the noted deviations and considering the Rec assay is not a standard method for DNA damage and repair.

B.6.4.1.36. *In vitro studies – Rec assay, study 2*

Data point:	CA 5.4.1/036
Report author	[redacted]
Report year	1978
Report title	The report of mutagenic study with bacteria for CP67573
Report No	ET-78-241
Document No	Not reported
Guidelines followed in study	No guideline followed, the study was conducted similar to U.S. EPA FIFRA Guidelines, Subdivision F
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	The DNA-damaging activity of glyphosate (CP67573, batch: XHJ-46, purity: 98.4%) was investigated in a DNA repair test with *Bacillus subtilis* strains H17 and M45. In a single experiment, the two bacterial strains were exposed to the test item at concentrations in the range of 20 – 2000 µg/disk. Solvent (water), negative (kanamycin, 10 µg/disk) and positive controls (mitomycin C, 0.1 µg/plate were included in the experiment. After overnight incubation at 37 °C, the growth inhibitory zones were measured for both strains.
Short description of results:	Precipitation of the test item was not investigated. Treatment with CP67573 did not induce any inhibitory zone in any of the two tester strains at any tested concentration. Solvent controls showed the expected results and negative controls treated with kanamycin induced growth inhibition zones of similar lengths in both strains. The positive control mitomycin C caused a

	marked difference in the length of the inhibitory zones, demonstrating the functionality of the test. Based on the results of the present study, glyphosate was negative for DNA-damaging activity in bacteria.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	The study was not conducted under GLP and not according to current testing guidelines. The test was performed in the absence of S9 mix only and no viability data (actual plate count) were provided. In addition, there were some reporting deficiencies. The study is therefore considered not acceptable. **Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since as there were many deficiencies.

B.6.4.1.37. *In vitro studies – Pol A+/- assay*

Data point:	CA 5.4.1/037
Report author	[redacted]
Report year	1993 (Finalisation of the English translation of report 87BME014 in Chinese language dated 03 Mar 1987)
Report title	Mutagenicity evaluation of glyphosate in *Escherichia coli.* DNA repair (pol A^+/A^-) assay.
Report No	87BME014-E
Document No	Not reported
Guidelines followed in study	No guideline followed. The study was conducted according to the main criteria of U.S. EPA FIFRA Guidelines, Subdivision F.
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	A DNA damage repair ($polA^+$/ $polA^-$) assay in *E. coli* strains W3110 ($polA^+$) and p3478 ($polA^-$) was performed to detect the mutagenic potential of glyphosate isopropylamine salt (code: SN-750721, purity: 64 %) on DNA damage level. In a single experiment with five replicates per condition, both bacterial strains were exposed to test item concentrations in the range of 0.1 – 10000 µg/mL. Solvent and positive controls (methyl methanesulfonate, 10 µL/disk) were included. After 48 hours of incubation at 37 °C, the length of inhibition zones around the disk was determined for both strains.
Short description of results:	Precipitation was not investigated in the study report. At the highest concentration of 10000 µg/mL, there was a strong growth inhibition observed in both strains, with a statistically significantly increased inhibition zone in strain p3478 ($polA^-$) when compared to strain W3110 ($polA^+$). There was no growth inhibition observed at lower test item concentrations. The solvent and positive control showed the expected results, demonstrating the sensitivity of the test. As growth inhibition induced by the test item was observed at one concentration only, the test result did not match the evaluation criteria for a positive result. Although growth inhibition was observed at the top dose level, the result was not confirmed in an independent experiment. Therefore, the test substance was considered equivocal for mutagenicity on DNA-damage level.
Reasons for why the study is not considered relevant/reliable or not	The study was not conducted under GLP and not according to current testing guidelines. The test was performed in the absence of metabolic activation only and no viability data (actual plate count) were provided. In addition, the title is misleading because not glyphosate acid but the

considered as key study:	isopropylamine salt has been tested. It is not clear whether the given purity refers to the contents of glyphosate in the formulation or the salt. A confirmatory experiment was not conducted even though there was some inhibition of cell growth at the top dose level. The study is therefore considered not acceptable. **Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since as there were many deficiencies. Not clear whether glyphosate has been tested.

B.6.4.1.38. *In vitro studies – SCE assay, study 1*

Data point:	CA 5.4.1/038
Report author	[redacted]
Report year	1993 (Finalisation of the English translation of report 87BMS013-E in Chinese language dated 03 Mar 1987)
Report title	Mutagenicity Evaluation of Glyphosate in Sister Chromatid Exchange Assay (SCE Test)
Report No	87BMS013-E
Document No	Not reported
Guidelines followed in study	No guideline followed. The study was conducted similarly to OECD 479 (1986)
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate isopropylamine salt (code: SN-750721, purity: 64 %) was tested in a sister chromatid exchange assay in Chinese hamster ovary (CHO) cells in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver S9 fraction). Untreated, solvent (DMSO) and positive controls (3.14 mM ethylmethane sulfonate without S9 mix and 0.01 mM cyclophosphamide with S9 mix) were included. A single experiment was performed. Duplicate cultures were exposed to test item concentrations in the range of 0.1 – 100 µg/mL for 1 hour, followed by 22 hours of incubation in the presence of 5-bromo-2-deoxyuridine. After 24 hours of incubation, the cells were harvested and a total of 30 metaphase cells per culture was scored for sister chromatid exchanges (SCEs) per chromosome.
Short description of results:	Cytotoxicity and solubility / precipitation were not investigated in the present study. Treatment with glyphosate isopropylamine salt did not induce a statistically significant increase in the frequency of SCEs per chromosome up to the highest tested concentration, neither in the presence nor in the absence of S9 mix. SCE frequencies of the untreated and solvent controls matched the acceptability criteria. A strong increase in the frequency of SCEs was noted for the positive control compounds, showing the activity of the S9 mix and demonstrating the sensitivity of the test. Under the experimental conditions reported, the test item was considered negative for sister chromatid exchange in CHO cells *in vitro*, with and without metabolic activation.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	The study was not conducted under GLP and not according to current testing guidelines. In addition, it is not clear whether the given purity refers to the contents of glyphosate in the formulation or the salt. The study is therefore considered not acceptable.

	Conclusion GRG: Considered invalid based on the information in RAR (2015). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since as there were many deficiencies. Not clear whether glyphosate has been tested.

B.6.4.1.39. *In vitro studies – SCE assay, study 2*

Data point:	CA 5.4.1/039
Report author	[redacted]
Report year	1990
Report title	Agrichem glyphosate active: OECD 479 sister chromatid exchange in human lymphocytes *in vitro*
Report No	300/2
Document No	Not reported
Guidelines followed in study	OECD TG 479 (1986)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate active (batch: 0190A, purity: not reported) was tested in a sister chromatid exchange assay in human lymphocytes (lymphocyte preparation not further specified). The cells were exposed to the test item, solvent (medium) or positive controls (ethyl methane sulfonate, 500 µg/mL without S9 mix and cyclophosphamide, 20 µg/mL with S9 mix) in the presence and absence of metabolic activation (Aroclor 1254-induced rat liver fraction). A single experiment was performed. Duplicate cultures were exposed to test item concentrations in the range of 78.125 – 2500 µg/mL in medium containing bromodeoxyuridine (BrdU). In the presence of S9 mix, the cells were exposed for 2 hours, followed by re-incubation in test substance-free medium for further 22 hours. In the absence of S9 mix, the cells were exposed for 24 hours. Afterwards, the mitotic cells were collected and prepared for staining. A total of 25 metaphase cells per culture (50 per condition) were scored for sister chromatid exchanges (SCE) per cell and per chromosome.
Short description of results:	Precipitation of the test substance in medium was not reported. Cytotoxicity (absence of metaphases) was observed at 1250 and 2500 µg/mL in the presence and absence of metabolic activation. Based on these findings, 625 µg/mL was selected as highest concentration for the evaluation of SCE. Treatment with glyphosate active did not induce a statistically significant, dose-related increase in the frequency of SCE per cell or per chromosome, neither in the presence, nor in the absence of metabolic activation. SCE values for the solvent and positive controls showed the expected results, indicating that the metabolic activation system was functional and demonstrating the sensitivity and validity of the test system. Under the conditions of the test, glyphosate active did not induce SCE in human lymphocytes *in vitro*, neither with nor without S9 mix.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	The study was considered not acceptable as only a single experiment was performed and the negative test result was not confirmed in an independent experiment. In addition, the purity of the test material was not stated. Furthermore, there were some reporting deficiencies, as lymphocyte preparation was not described in detail (remaining uncertainty about exposure of isolated lymphocytes or whole blood cell culture). The study is therefore considered invalid. **Conclusion GRG:** Considered invalid based on the information in RAR (2015). Category 3b.

	Conclusion AGG: In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation since as there were a lot of deficiencies.

B.6.4.1.40. *In vitro studies – V79 HPRT Gene Mutation*

Data point	5.4.1/040
Report author	[redacted]
Report year	2021
Report title	Glyphosate: V79 HPRT Gene Mutation Assay
Report No	8441968 (Covance Laboratories Ltd.)
Document No	CV-2020-0234
Guidelines followed in study	OECD 476 (2016), Council Regulation (EC) No. 440/2008 method B17 (2008), US EPA OPPTS 870.5300 (1998)
Deviations from current test guideline OECD 476 (2016)	None
Previous evaluation	No, not previously submitted
GLP/Officially recognised testing facilities	Yes/Yes The study was performed at Covance Laboratories Limited, Shardlow, UK.
Acceptability/Reliability	**Conclusion GRG:** Yes/yes; Category 1 **Conclusion AGG**: The study is considered to be acceptable.

Executive summary

Glyphosate (AZM30320T0, purity 91.8%) was tested *in vitro* for its ability to induce forward mutations in mammalian cells by assessing the mutation of the HPRT locus in Chinese hamster V79 cells in the presence and absence of S9 mix (phenobarbital and 5,6-benzoflavone-induced rat liver S9 fraction). Test item concentrations were selected based on the results of a preliminary cytotoxicity test, in which no cytotoxicity was evident up to the highest tested concentration of 1691 µg/mL (corresponding to the limit test concentration of 10 mM).

In the main experiment, V79 cells were exposed to test item concentrations in the range of 105–1691 µg/mL for 4 hours in the presence and absence of S9 mix. Solvent (water) and positive controls (9,10-dimethyl-1,2-benzanthracene (DMBA) (2 µg/mL) with S9 mix and ethylmethane sulfonate (EMS) (500 and 750 µg/mL) without S9 mix) were included. Following exposure with and without S9 mix, the cells were re-plated and incubated for 7 days to allow expression of the mutant phenotype. The expression period was followed by a selection period, in which the cells were cultivated on 6-thioguanidine-enriched medium for 7 days. Cytotoxicity was assessed as relative survival and relative viability after the expression and selection period, respectively.

There was no precipitation of the test item in solvent and in culture medium, neither in the presence nor absence of S9 mix. A reduction in pH of 1.23 pH-units was observed at 1691 µg/mL. There was no cytotoxicity at any concentration, neither in the presence, nor the absence of S9 mix.

There was a statistically significant increase in mutant frequency in the absence of S9 mix at 845.5 and 1268.3 µg/mL, however, the increase was not dose-dependent and fell within the historical control range for the solvent control.

Mutant frequencies of the solvent control cultures remained within the range of the laboratory's historical control data. The positive control mutagens EMS and DMBA showed the expected results, thereby demonstrating the functionality of the S9 mix and the sensitivity of the test.

Based on the experimental findings and under the conditions of the test, there was no evidence for gene mutation in mammalian cells *in vitro*, neither in the presence nor in the absence of metabolic activation.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Glyphosate (N-phosphonomethylglycine)

Identification:	Not specified
Description:	White crystalline solid
Lot/Batch number:	AZM30320T0
Purity:	91.8 w/w (Certificate of Analysis)
Stability of test compound:	The stability of the test item at storage conditions (at 20 – 25 °C in a tightly closed container in a dry and well ventilated, dark place) was guaranteed until the expiry date 12 Oct 2023. The stability of the test substance in vehicle was verified by analytical methods for up to 24 h.

2. Control material:

Negative control:	The negative control was actually the solvent control.
Solvent (vehicle) control:	Water
Positive control:	- S9 mix: Ethylmethane sulfonate (EMS), 500 and 750 µg/mL in DMSO + S9 mix: 9,10-Dimethyl-1,2-benzanthracene (DMBA), 2 µg/mL in DMSO

3. Metabolic activation:

S9 mix was purchased from [redacted] (Lot no. 4222). The S9 was pre-tested for acceptability by the supplier prior to purchase and was supplied with a relevant "Quality Control & Production Certificate" which is presented in the study report. The liver homogenate was obtained from the livers of male Sprague-Dawley rats at 5 – 6 weeks of age, weighing 175 – 199 g. The animals were induced with phenobarbital and 5,6-benzoflavone. The S9 homogenate was stored in liquid nitrogen until use. Immediately prior to use the S9 liver homogenate was thawed and mixed with co-factors as follows:

S9 mix component	Concentration	Unit
Phosphate buffer	Not specified	
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	20%	

The protein content of the S9 mix was adjusted to 20 mg/mL prior to use. The final concentration of S9 mix in the treatment medium was 2%.

4. Test organism:

V79 cells were obtained from Harlan CCR (Roßdorf, Germany). The cells were periodically checked for the absence of mycoplasma contamination and the spontaneous mutation rate was continuously monitored.

5. Cell culture media:

Cultivation medium (MEM-FBS):	Eagles Minimal Essential (MEM) (supplemented with sodium bicarbonate, L-glutamine, penicillin/streptomycin, amphotericin B, HEPES buffer and 10% fetal bovine serum (FBS))
Treatment medium ± S9):	Serum-free Minimal Essential Medium (MEM)
Selection medium:	Cultivation medium (MEM-FBS) supplemented with 11 µg/mL 6-thioguanidine (6-TG)
Incubation:	At 37 °C in a humidified atmosphere containing 5% CO_2

6. Locus examined: Hypoxanthin guanine phosphoribosyltransferase (HPRT)

7. Test concentrations and number of replicates:

a) Preliminary cytotoxicity test

Metabolic activation	Duration of exposure	Concentrations	Replicates
± S9 mix	4 h	13.21, 26.42, 52.84, 105.69, 211.38, 422.75, 845.5, 1268.3, 1691 µg/mL	Single culture

b) Main gene mutation assay

Metabolic activation	Duration of exposure	Concentrations	Replicates
± S9 mix	4 h	0, 105.69, 211.38, 422.75, 845.5, 1268.3, 1691 µg/mL	Duplicate

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 08 Jul – 27 Aug 2020
Finalisation date: 18 February 2021

2. Preliminary cytotoxicity test:
A preliminary test was performed to identify suitable dose levels for the main mutagenicity study. Cell cultures were established identically to the performance in the main mutagenicity test described below. The cells were exposed to nine test item concentrations in the range of 13.21 – 1691 µg/mL for 4 hours in the presence and absence of S9 mix. For each condition, one flask was used for treatment. Following exposure, the cells were washed twice with phosphate buffered saline (PBS), trypsinised and plated in triplicates to determine a relative cloning efficiency. After 7 days of incubation at 37 °C, the cells were fixed, stained and counted.
Based on the results of the preliminary test, the test item concentrations for the main mutagenicity test were selected.

3. Main mutation assay:
Pre-treatment of cells:
For each condition, 2 x 10^6 cells were seeded into 225 cm² flasks in cultivation medium 2 days prior to treatment. This resulted in 20 x 10^6 available cells at dosing. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Treatment:
A single experiment was performed. Duplicate cultures each were exposed to the test item, positive (DMBA, 2 µg/mL in the presence of S9 mix and EMS, 500 and 700 µg/mL in the absence of S9 mix) or solvent (water) controls for 4 hours in the presence and absence of S9 mix. In the presence and absence of S9 mix, test item concentrations were in the range of 105.69 – 1691 µg/mL.
Following exposure, the cells were washed twice with PBS, trypsinised and 200 cells were seeded in 25 cm² flasks in triplicates for the determination of cell survival (cloning efficiency 1, CE_1) and subsequent calculation of relative survival (RS). A total of 2 x 10^6 cells were re-cultured in a 225 cm^2 flask for expression of the mutant phenotype.

Expression period:
After treatment, the cells were re-cultured in cultivation medium for a 7-day expression period, including sub-cultivation on Days 2 and 5. After the expression period, the cells were harvested by trypsinisation and the cultures were divided. Triplicate cultures of 200 cells/dish were seeded in 25 cm² flasks for the determination of viability (cloning efficiency 2, CE_2). Ten replicate plates with 2 x 10^5 cells were seeded for the selection of mutants.

Selection period:

For the selection of mutants, ten culture dishes with 2 x 10^5 cells were seeded in medium enriched with 11 µg/mL 6-thioguanidine (selection medium). After an incubation period of 7 days, the colonies were fixed, stained and counted.

4. Cytotoxicity:

Day 0 viability

The Day 0 viability of test item-treated cells relative to solvent controls was determined in parallel to the mutagenicity test. At the end of the exposure period, a sample of each cell culture was collected to assess survival of the cells. Triplicates of 200 cells/25 cm² dish were seeded and incubated for 6 days. Afterwards, the colonies were fixed, stained and counted. Based on the Day 0 viability, the relative survival (RS) was calculated.

Day 7 viability

The Day 7 viability was determined in parallel to the selection of mutants. After the expression period, triplicates of 200 cells/25 cm² dish were seeded in medium without 6-thioguanine to assess cell viability. After an incubation period of 7 days, the colonies were fixed, stained and counted.

5. Evaluation and calculations:

Determination of cytotoxicity

Cloning efficiency (CE, termed Day x viability in the study report, %):

$$\text{CE\%} = \frac{\text{Mean number of colonies in the test group}}{\text{Total number of seeded cells in the test group (200)}} \times 100$$

$$\text{Adjusted CE: Adjusted CE} = \text{CE\%} \times \frac{\text{Number of cells at the end of treatment}}{\text{Number of cells at the beginning of treatment}}$$

$$\text{Relative survival (RS, \% of control): RS} = \frac{\text{Adjusted CE in treated culture}}{\text{Adjusted CE in the solvent control}} \times 100$$

Determination of mutant frequency

The mutant frequency is defined by the cloning efficiency of mutant colonies in selective medium divided by the cloning efficiency in non-selective medium measured for the same culture at the time of selection was calculated for each sample.

The mutant frequency (MF) for each dose was calculated as follows:

$$\text{MF} = \frac{\text{Total number of mutant colonies}}{2}$$

The mutation frequency/10^{-6} survival rate (MFS 10^{-6}) was calculated regarding the values of CE_2:

$$\text{MFS } 10^{-6} = \frac{\text{MF}}{\text{Day 7 CE\%}} \times 100$$

6. Statistics:

Statistical analysis was performed, if there was an increase of mutant frequencies in any dose level. In this case, comparisons were made between the solvent control value and each individual dose level, using Student's t-test. To assess the dose-relationship, a linear regression model was used. An arcsin square-root transformation was applied to the mutant frequency per survivor (excluding positive controls). A linear regression model was then applied to these transformed values with dose values fitted as the explanatory variable. The F-value from the model was assessed at the 5% statistical significance level.

7.Acceptance criteria:

The test was considered valid if the following criteria were met:

- The spontaneous mutant frequency of the solvent control was within the range of the laboratory's historical control data.
- The positive controls caused an increase in mutation frequency comparable to the historical control data and statistically significantly higher than the solvent control.
- The criteria for selection of the maximum concentration as specified in the guideline have been met.

- Two experimental conditions (with and without metabolic activation) were tested unless one resulted in a positive response.
- Adequate numbers of cells and concentrations were analysable.
- A minimum of 4 analysed duplicate dose levels was considered necessary in order to accept a single assay for evaluation of the test item.

8. Evaluation criteria:

A test item was judged positive[6] for gene mutation in mammalian cells if the following criteria were met:

- A significant increase in mutant frequency was observed in at least one concentration when compared to the solvent control.
- The increase was concentration dependent.
- The results were outside the range of the historical solvent control data for the test item concentrations.

A test item was judged negative[7] for gene mutation in mammalian cells if the following criteria were met:

- The test item did not increase the mutation frequency compared to the solvent control under any condition.
- There was no concentration dependent increase.
- The results of the test item concentration were within the range of the historical solvent control data.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Dose formulation analysis showed that the applied test item concentrations were accurate, within acceptable limits and stable at room temperature in the light. The analysed concentrations ranged from 88 – 100% (after 4 hours of storage) and 96 – 101% (after 24 hours of storage) of the initial concentration. All mixtures were homogenous.

B. CYTOTOXICITY

In the preliminary cytotoxicity test, no concentration-dependent decrease of RS was found with and without metabolic activation. A reduction in relative survival was seen at 211.38 µg/mL without S9 mix, however, the finding was not considered biological relevant because it was not dose-dependent. The maximum dose was the 10 mM limit concentration (equivalent to 1691 µg/mL).

In the main test, no cytotoxicity was observed. Neither RS nor CE_2 were affected by treatment with glyphosate at any test item concentration with and without metabolic activation.

C. SOLUBILITY

In the preliminary cytotoxicity test as well as in the main mutation test, precipitation of the test item in medium was not observed up to the highest concentration of 1691 µg/mL, neither in the presence or absence of S9 mix.

A change in the pH of more than 1 pH unit was observed at the highest concentration of 1691 µg/mL (decrease of 1.23 pH units) only. Osmolality did not change more than 50 mOSm at any concentration. Based on these findings, the highest test item concentrations for the main mutagenicity study was 1691 µg/mL. The pH and osmolality readings are presented in the following table:

Concentration (µg/mL)	0.00	6.61	13.21	26.42	52.84	105.69	211.38	422.75	845.5	1268.3	1691
pH	7.51	7.50	7.51	7.49	7.47	7.45	7.35	7.25	7.00	6.89	6.28
Osmolality mOsm	284	282	-	309	-	286	-	287	-	291	280

- = not determined

[6] The RMS notes that according to the OECD test guideline 476 a test item is judged clearly positive if the criteria stated are met.

[7] The RMS notes that according to the OECD test guideline 476 a test item is judged clearly negative if the criteria stated are met.

D. MUTANT FREQUENCY

Statistically significantly increased mutant frequencies were observed after 4 hour treatment with glyphosate at concentrations of 845.5 and 1268.3 µg/mL without S9 mix. The findings were considered of no biological relevance because the values were in the historical 95% control limits, no dose dependency was observable and no statistically significant increase was found when analysed with a linear regression model. All results fell into the normal range of solvent controls. There was no statistically significant increase in the mutant frequency of cultures exposed in the presence of S9 mix.

Mutant frequencies of the solvent control cultures remained within the range of the laboratories historical control data. The positive control mutagens EMS and DMBA showed the expected results, thereby demonstrating the functionality of the S9 mix and the sensitivity of the test.

Table B.6.4.1.40-1: Results of the HPRT gene mutation assay in mammalian cells with Glyphosate ([redacted], 2021): 4-hour incubation without metabolic activation

Concentration (µg/mL)		Day 0 viability				Day 7 viability[#]			Day 7 Mutant frequency			
		% CE	CE	RS	**Mean RS**	% CE	% Control	**Mean % control**	MF	MFS 10^{-6}	SD	**Group MFS 10^{-6}**
Vehicle (Water)												
0	A	90.5	89.0	100	**100**	81.7	100	**100**	6.5	8.0	1.61	**9**
	B	84.8	82.5	100					9	11.0		
HCD mean ± SD										13.1 ± 3.5		
Range (95% control limits)										6.2 – 20.0		
Test item												
105.69	A	94.7	93.1	104.6	**102**	65.2	79.8	**87**	4.5	6.9	0.85	**8**
	B	83.8	81.6	98.8		76.2	93.3		6.5	8.5		
211.38	A	86.2	84.7	95.2	**99**	102.0	124.9	**117**	9.5	9.3	1.23	**11**
	B	88.0	85.6	103.7		88.8	108.8		11.0	12.4		
422.75	A	83.3	81.9	92.1	**89**	91.5	112.0	**115**	9.5	10.4	1.47	**10**
	B	72.3	70.4	85.3		96.3	118.0		8.5	8.8		
845.5	A	87.2	85.7	96.3	**93**	96.2	117.8	**109**	13.0	13.5	0.97	**16****
	B	76.3	74.3	90.0		82.3	100.8		14.5	17.6		
1268.3	A	79.2	77.8	87.5	**90**	98.8	121.0	**113**	11.5	11.6	1.46	**14***
	B	77.8	75.7	91.7		86.5	105.9		15.0	17.3		
1691	A	81.2	79.8	89.7	**95**	94.2	115.3	**106**	9.5	10.1	0.88	**10**
	B	85.5	83.2	100.8		78.2	95.7		7.0	9.0		
Positive control (EMS)												
500	A	84.5	83.1	93.4	**94**	85.0	104.1	**106**	218	256.5	8.89	**222*****
	B	79.5	77.3	93.7		87.5	107.1		163.5	186.9		
HCD mean ± SD										281.9 ± 86.0		
Range (95% control limits)										109.8 – 454.0		
750	A	59.2	58.2	65.4	**69**	73.2	89.6	**78**	324	442.8	8.67	**474*****
	B	61.2	59.5	72.1		53.7	65.7		271	505.0		
HCD mean ± SD										454.7 ± 154.3		
Range (95% control limits)										146.2 – 763.2		

EMS: Ethylmethane sulfonate; CE: Cloning efficiency; RS: relative survival; MF: Mutant frequency; MFS: Mutant frequency per survivor;
Statistical significance at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ level;

HCD: historical control data generated in the testing laboratory (time frame not specified), as available from the study report (n=24 and n=23 for the vehicle and positive controls, respectively);
[#] The Day 7 vehicle control viability flasks were lost due to a technical error and so the cloning efficiency was based on the B replicate only.

Table B.6.4.1.40-2: Results of the HPRT gene mutation assay in mammalian cells with Glyphosate [redacted], 2021): 4-hour incubation with metabolic activation

Concentration (µg/mL)		Day 0 viability[#]				Day 7 viability			Day 7 Mutant frequency			
		% CE	CE	RS	**Mean RS**	% CE	% Control	**Mean % control**	MF	MFS 10^{-6}	SD	**Group MFS 10^{-6}**
Vehicle (Water)												
0	A	79.5	76.6	100	**100**	85.7	100	**100**	2.5	2.9	0.99	**5**
	B	65.0	57.3	100		86.2	100		6	7.0		
HCD mean ± SD										12.5 ± 3.0		
Range (95% control limits)										6.6 - 18.5		
Test item												
105.69	A	66.2	63.7	83.2	**92**	90.7	105.8	**106**	3	3.3	1.65	**10**
	B	65.7	57.9	101.0		91.8	106.6		14.5	15.8		
211.38	A	70.5	67.9	88.7	**97**	91.3	106.6	**109**	8	8.8	1.41	**9**
	B	68.3	60.3	105.1		95.5	110.8		9.5	9.9		
422.75	A	49.5	47.7	62.3	**62**	85.8	100.2	**106**	4	4.7	1.41	**9**
	B					95.7	111.0		13.5	14.1		
845.5	A	69.2	66.6	87.0	**111**	96.0	112.1	**108**	6	6.3	0.76	**6**
	B	88.3	77.9	135.9		89.2	103.5		4.5	5.0		
1268.3	A	63.7	61.3	80.1	**101**	87.2	101.8	**104**	11.5	13.2	1.24	**10**
	B	79.2	69.8	121.8		91.8	106.6		6.5	7.1		
1691	A	32.5	31.3	40.9	**65**	85.0	99.2	**102**	5	5.9	1.00	**8**
	B	58.3	51.4	89.6		90.5	105.0		9.5	10.5		
Positive control (DMBA)												
2	A	37.5	36.1	47.2	**60**	72.0	84.0	**88**	316.5	439.6	13.23	**479*** **
	B	46.8	41.3	72.1		78.8	91.5		408.5	518.2		
HCD mean ± SD										601.2 ± 228.0		
Range (95% control limits)										145.3 - 1057.1		

DMBA: Dimethyl benzanthracene; CE: Cloning efficiency; RS: relative survival; MF: Mutant frequency; MFS: Mutant frequency per survivor;
Statistical significance at ** $p < 0.01$ and *** $p < 0.001$ level;

HCD: historical control data generated in the testing laboratory (time frame not specified), as available from the study report (n=24 and n=23 for the vehicle and positive controls, respectively);
[#] The Day 0 viability flasks for the B replicate at the 422.75 µg/mL concentration were lost due to a technical error and so the cloning efficiency was based on the A replicate only.

III. CONCLUSIONS

Based on the experimental findings and under the conditions of the test, glyphosate did not induce any toxicologically significant or dose-related increase of gene mutations in the HPRT locus of V79 cells, neither in the presence nor in the absence of metabolic activation. It is therefore considered negative for mutagenicity in mammalian cells *in vitro*.

Assessment and conclusion by applicant:

Under the conditions of the test, glyphosate was negative for mutagenicity at the HPRT locus in V79 cells with and without metabolic activation.

The study was conducted in compliance with GLP and according to OECD guideline 476 (2016). There were only minor deviations when compared to OECD 476 (2016), which were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:

It is agreed with the applicant's conclusion. Glyphosate (batch AZM30320T0; purity 91.8%) was negative for gene mutation at the HPRT gene locus of V79 cells with and without metabolic activation under the conditions of this *in vitro* cell gene mutation assay.

B.6.4.1.41. *In vitro studies – Micronucleus test in human peripheral lymphocytes*

Data point	5.4.1/041
Report author	[redacted]
Report year	2021
Report title	Glyphosate: Micronucleus Test in Human Lymphocytes *in vitro*
Report No	8441969 (Monsanto)
Document No	CV-2020-0236
Guidelines followed in study	OECD 487 (2016)
Deviations from current test guideline OECD 487 (2016)	Modification of the suggested extended treatment schedule (see study summary). The RMS agrees with the justification for this modification.
Previous evaluation	No, not previously submitted.
GLP/Officially recognised testing facilities	Yes/yes The study was conducted at Covance Laboratories Ltd., Shardlow, Derbyshire, United Kingdom
Acceptability/Reliability	**Conclusion GRG:** Yes/yes, Category 1 **Conclusion AGG:** The study is considered to be acceptable.

Executive Summary

Glyphosate (batch: AZM30320T0, purity: 91.8%) was investigated for induction in the frequency of binucleated cells with micronuclei in human peripheral lymphocytes in the presence and absence of metabolic activation (phenobarbital and 5,6-benzoflavone-induced rat liver S9 fraction). Based on the results of a preliminary cytotoxicity test, dose levels for the main micronucles test were in the range of 105.69 – 1691 µg/mL (corresponding to the limit test concentration of 10 mM).

Duplicate cultures were exposed for 4 hours in the absence or presence of S9 mix and for 24 hours in the absence of S9 mix. Solvent (water) and clastogenic (0.2 µg/mL mitomycin C for 4 hour exposure without S9 mix, 6 µg/mL cyclophosphamide for 4 hour exposure with S9 mix) and aneugenic positive controls (0.075 µg/mL demecolcine for 24 hour exposure without S9 mix) were included. After exposure, cells were cultivated in cytochalasin B-enriched medium for 24 hours prior to harvest.

A total of at least 2000 binucleated cells (4000 for solvent controls) were scored for the presence of micronuclei. In addition, cytotoxicity was evaluated as cytokinesis block proliferation index (CBPI).

There was no precipitation of the test substance in culture medium observed at the end of treatment, not for any concentration, neither with nor without S9 mix.

Cytotoxicity, evident as 34% cytostasis, was noted after 24 hours of exposure at 1691 µg/mL without S9 mix. There was no marked cytotoxicity after 4 hours of exposure with or without metabolic activation.

There was a small but statistically significant increase in the frequency of binucleated cells observed after 24 hours of exposure in the absence of S9 mix at the lowest dose level examined (422.75 µg/mL). The increase was within the range of the laboratory's historical control data and, in the absence of any dose-response relationship, considered to be without any biological relevance. There was no increase in the frequency of binucleated cells at any other concentration or after 4 hours of exposure, neither in the presence nor absence of S9 mix.

In both experiments, the frequency of micronucleated cells was within the range of the laboratory's historical control data for the solvent control.

The clastogenic and aneugenic positive controls induced statistically significant increases in the frequency of micronucleated cells in all experiments, demonstrating the functionality of the metabolic activation system and the validity of the assay.

Based on the experimental findings, glyphosate was negative for the induction of micronuclei in human peripheral lymphocytes *in vitro*, in the presence and in the absence of metabolic activation.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: N-phosphonomethylglycine (CAS No. 1071-83-6)

Identification:	Glyphosate
Description:	White crystalline solid
Lot/Batch #:	AZM30320T0
Purity:	91.8% w/w
Stability of test compound:	The stability of the test item under storage conditions (at room temperature in the dark) was guaranteed until 12 Oct 2023. The stability of the test item in solvent (vehicle) was verified by analytical methods.
Solvent (vehicle) used:	Water

2. Control materials

Negative control:	The solvent control is actually the negative control.
Solvent (vehicle) control:	Water
Positive controls:	Please refer to table below.

Clastogenic positive controls	
Short-term treatment (4-hour exposure)	
- S9	Mitomycin C (MMC), 0.2 µg/mL in Minimal essential medium
+ S9	Cyclophosphamide (CP), 6.0 µg/mL in DMSO
Aneugenic positive controls	
Long-term treatment (24-hour exposure)	
- S9	Demecolcine (DC), 0.075 µg/mL in sterile distilled water

Demecolcine (DC) is not one of the suggested positive control substances listed in the OECD 487 guideline but the substances are recommendations only, and DC is a derivative of Colchicine, one of the recommended substances. There is sufficient laboratory historical control data to demonstrate its effectiveness and suitability as an aneugen.

3. Metabolic activation:

S9 mix was purchased from [redacted] (Lot no 4222, expiry date 12 March 2022) and obtained from the livers of male Sprague-Dawley rats, that were induced with phenobarbital and 5,6-benzoflavone. The animals were 5-6 weeks of age and 175 – 199 g of weight. The protein content was adjusted to 20 mg/mL prior to use. The S9 was pre-tested for acceptability by the supplier prior to purchase and was supplied with a relevant "Quality Control & Production Certificate" which is presented in the study report.

Prior to each experiment, co-factors were added to the S9 mix containing the following components:

S9 mix component	Concentration	Unit
Sodium orthophosphate buffer (pH 7.4)	100	mM
KCl	33	mM
NADPH-generating system		
Glucose 6-phosphate	5	mM
NADP	5	mM
$MgCl_2$	8	mM
S9	20	%

The final concentration of S9 the cell culture medium was 2%.

4. Test organism:

Human peripheral blood was obtained by venepuncture from a healthy, non-smoking volunteer (female, 28 years of age for the preliminary toxicity test and male, 25 years of age for main experiment) and collected in heparinised vessels. The cells were reported to have an average generation time of approximately 16 hours.

5. Cell culture:

Complete culture and treatment medium: Eagles minimal essential medium with HEPES (MEM) supplemented with 10% foetal bovine serum (FBS), penicillin/ streptomycin solution, L-glutamine and amphotericin B.

Incubation: At 37 °C and 5% CO_2 in humidified air.

Cell culture establishment prior to exposure

Approximately 0.5 mL of heparinised whole blood was cultured with 8.25 – 9.27 mL MEM supplemented with 10% FBS, 0.1 mL lithium heparin and 0.1 mL phytohaemagglutinin (PHA) for 48 hours prior to treatment.

6. Test concentrations and number of replicates:

a) Preliminary toxicity test

Metabolic activation	Duration of exposure (Fixation)	Concentrations	Replicates
± S9 mix	4 h (28 h)	13.21, 26.42, 52.84, 105.69, 211.38, 422.75, 845.5, 1268.25 and 1691 µg/mL	Single culture (duplicate for control)
-S9 mix	24 h (48 h)	13.21, 26.42, 52.84, 105.69, 211.38, 422.75, 845.5, 1268.25 and 1691 µg/mL	Single culture (duplicate for control)

b) Main micronucleus test

Metabolic activation	Duration of exposure (Fixation)	Concentrations	Replicates
± S9 mix	4 h (28 h)	105.69, 211.38, 422.75*, 845.5*, 1268.25* and 1691* µg/mL	Duplicate (4 replicates for control)
- S9 mix	24 h (48 h)	105.69, 211.38, 422.75*, 845.5*, 1268.25* and 1691* µg/mL	Duplicate (4 replicates for control)

*: Concentrations selected for microscopic analysis of micronucleated frequencies.

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 03 Jul – 18 Aug 2020
Finalisation date: 18 February 2021

2. Preliminary cytotoxicity test:

In a preliminary cytotoxicity test, human lymphocytes were treated with the test item at concentrations of 13.21 to 1691 µg/mL both, with and without metabolic activation under the same conditions as in the main mutagenicity test (described below). The maximum dose was the maximum recommended dose level by the guideline, equivalent to 10 mM concentration. One single cell culture per condition was exposed to the test item for 4 hours in the presence and absence of S9 mix or for 24 hours in the absence of S9 mix. Cells were prepared about 24 hours following the completion of exposure.

Using a qualitative microscopic evaluation of the microscope slide preparations from each treatment culture, appropriate dose levels were selected for the evaluation of the frequency of binucleate cells and to calculate the cytokinesis block proliferation index (CBPI). The CBPI data were used to estimate test item toxicity and for selection of the dose levels for the exposure groups of the main experiment.

3. Micronucleus test:

Treatment and cytokinesis block: About 48 hours after cell culture establishment, approximately 9 mL of the culture medium was removed and replaced by fresh culture medium. 1 mL of the appropriate test item, vehicle or positive control solution was added.
Duplicate cultures were exposed for 4 hours in the absence or presence of S9 mix and for 24 hours in the absence of S9 mix at test item concentrations in the range of 105.69 – 1691 µg/mL. Solvent (water) and positive controls (0.2 µg/mL mitomycin C for 4 hour exposure without S9 mix, 6 µg/mL cyclophosphamide for 4 hour exposure with S9 mix and 0.075 µg/mL demecolcine for 24 hour exposure without S9 mix) were included.
Following exposure, cells were centrifuged and washed in fresh culture medium. Thereafter, cells were incubated in fresh culture medium including 4.5 µg/mL cytochalasin B for another 24 hours.

The RMS notes the following: The extended exposure for the extended treatment (24 hours without S9 mix) detailed above is a modification of the suggested cell treatment schedule in the OECD Guideline 487. According to the study director, this is considered to be an acceptable alternative because it avoids any potential interaction between cytochalasin B and the test item during exposure and any effect this may have on the activity or response. Additionally, the study director stated that as the stability or reactivity of the test item is unknown prior to the start of the study this modification of the schedule is considered more effective and reproducible due to the in-house observations on human lymphocytes and their particular growth characteristics in this study type and also the significant laboratory historical control data using the above format. The RMS agrees with this justification on the modification of the suggested cell treatment schedule.

Cell harvest: At the end of the incubation (24 hours after end of exposure), the culture medium was removed. The cells were treated with a mild hypotonic solution (0.0375 M KCl) before

being fixed with fresh methanol/glacial acetic acid (19:1 v/v).

Slide preparation: Cells were re-suspended in fresh fixative, centrifuged, re-suspended in a small amount of fixative and the suspension was dropped onto clean, wet microscope slides and left to air dry with gentle warming. Afterwards, cells were stained with 5% Giemsa.

Metaphase analysis: A total number of at least 2000 binucleated cells per condition (1000 per culture) were examined by microscopy and scored for the presence of micronuclei. For the vehicle controls, a total of 4000 binucleated cells were scored.

Cytotoxicity: The cytokinesis block proliferation index (CBPI) was determined from 500 cells. In addition, the percentage of cytostasis was determined, which indicates the inhibition of cell growth in treated cultures in comparison to control cultures. Based on the current OECD guideline 487 (2016), cytotoxicity should not exceed the limit of 55 ± 5%.

Formulas: Calculation of the Cytokinesis Block Proliferation Index (CBPI):

$$CBPI = \frac{(c1\ x\ 1) + (c2\ x\ 2) + (cm\ x\ 3)}{n}$$

c_1: mononucleate cells
c_2: binucleate cells
c_m: multinucleate cells
n: total number of cells

Calculation of cytostasis:

$$\%\ Cytostasis = 100 - 100\ x \frac{(CBPI\ treated\ culture - 1)}{(CBPI\ control\ culture - 1)}$$

4. Statistics:

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the non-parametric Chi-square test. The p-value was used as a limit in judging for significance levels in comparison with the corresponding negative control. The dose-relationship (trend-test) was assessed using a linear regression model. An arcsin square-root transformation was applied to the percentage of binucleated cells containing micronuclei (excluding positive controls). A linear regression model was then applied to these transformed values with dose values fitted as the explanatory variable. The F-value from the model was assessed at the 5% statistical significance level.

5. Acceptance criteria:

The study was considered valid if the following criteria were met:

- The concurrent negative control was considered acceptable for addition to the laboratory historical negative control data range.
- All the positive control chemicals induced positive responses that were compatible with those in the laboratory historical positive control data range and produced a statistically significant increase when compared with the concurrent negative control. Acceptable positive responses demonstrated the validity of the experiment and the integrity of the S9-mix.
- Cell proliferation criteria in the solvent control were considered to be acceptable.
- The study was performed using all three exposure conditions using a top concentration which meets the requirements of the current testing guideline.
- The required number of cells and concentrations were analysed.

6. Evaluation criteria:

A test substance was considered positive for induction of micronuclei when the following criteria were met:

- At least one of the test concentrations exhibited a statistically significant increase compared with the concurrent negative control.

- The increase was dose-related in at least one experimental condition when evaluated with an appropriate trend test.
- The results were substantially outside the range of the laboratory historical negative control data.

A test item was considered negative for induction of micronuclei when the following criteria were met:

- None of the test concentrations exhibited a statistically significant increase compared with the concurrent negative control.
- There was no dose-related increase when evaluated with an appropriate trend test.
- The results in all evaluated dose groups were within the range of the vehicle laboratory historical control data.

1. RESULTS AND DISCUSSION

I. ANALYTICAL DETERMINATIONS

Dose formulation analysis performed for the main experiment demonstrated that the test item formulations were accurate and within acceptable limits (87 - 104% of the nominal concentrations). Stability and homogeneity were evaluated as part of a different study (method development study report no. 8442134, KCA 4.1.2-226). The test item formulations were shown to be stable for up to 24-hours.

J. CYTOTOXICITY

In the preliminary cytotoxicity test, slight cytotoxicity was observed after 24 hours of exposure in the absence of S9 mix (45% cytostasis at 1691 µg/mL).

In the main micronucleus test, cytotoxicity of about 34% cytostasis was noted after 24 hours of exposure at 1691 µg/mL without S9 mix. There was no marked cytotoxicity after 4 hours of exposure with or without metabolic activation.

At 1691 µg/mL, there was further a small decrease in pH (-1.23 pH units). It was considered acceptable to use this dose level in the study since the change in pH was only slightly above one pH unit and it meant that the test item was tested to the maximum recommended dose level. There was no change on osmolality by more than 50 mOsm at any dose level tested.

K. SOLUBILITY

There was no precipitation of the test item in blood-free culture medium observed for any concentration at the end of treatment, neither with, nor without S9 mix.

L. MICRONUCLEUS TEST

There was a small but statistically significant increase in the frequency of binucleated cells observed after 24 hours of exposure in the absence of S9 mix at the lowest dose level examined (422.75 µg/mL). The increase was within the range of the laboratory's historical control data and, in the absence of any dose-response relationship, considered to be without any biological relevance. There was no increase in the frequency of binucleated cells at any other concentration or after 4 hours of exposure, neither in the presence nor absence of S9 mix.

The vehicle control cultures showed frequencies of binucleated cells within the accepted range and the clastogenic and aneugenic positive controls induced statistically significant increases in the frequency of micronucleated cells in all experiments, demonstrating the functionality of the metabolic activation system and the validity of the assay.

Table B.6.4.1.41-1: Glyphosate - Summary of genotoxicity data obtained in the micronucleus test in human lymphocytes *in vitro* ([redacted] 2021)

Compound	Concentration [µg/mL]	No. of binucleated cells scored	Genotoxicity	Cytotoxicity	
			% Binucleated cells containing micronuclei[a]	Mean CBPI	Mean cytostasis [%]
Without metabolic activation; 4-hours treatment					
Solvent					
Water	0	4000	**0.18**	1.26	0
HCD[#] mean ± SD			0.40 ± 0.16		
Range (95% control limits)			*0.08 – 0.72*		
Test item					
	422.75	2000	**0.10**	1.33	0[x]
	845.50	2000	**0.20**	1.26	1
	1268.25	2000	**0.10**	1.25	3
	1691.00	2000	**0.10**	1.33	0[x]
Positive control					
MMC	0.20	2000	**3.45*** **	1.22	15
HCD[#] mean ± SD			3.71 ± 1.25		
Range (95% control limits)			*1.21 – 6.21*		
With metabolic activation; 4-hours treatment					
Solvent					
Water	0	4000	**0.15**	1.47	0
HCD[#] mean ± SD			0.39 ± 0.21		
Range (95% control limits)			*0 - 0.81*		
Test item					
	422.75	2000	**0.00**	1.50	0[x]
	845.50	2000	**0.10**	1.45	4
	1268.25	2000	**0.05**	1.41	13
	1691.00	2000	**0.10**	1.40	15
Positive control					
CP	6.00	2000	**2.50*** **	1.23	51
HCD[#] mean ± SD			2.18 ± 0.61		
Range (95% control limits)			*0.96 – 3.40*		
Without metabolic activation; 24-hours treatment					
Solvent					
Water	0	4000	**0.10**	1.76	0

Compound	Concentration [µg/mL]	No. of binucleated cells scored	Genotoxicity	Cytotoxicity	
			% Binucleated cells containing micronuclei[a]	Mean CBPI	Mean cytostasis [%]
HCD[#] mean ± SD			0.35 ± 0.17		
Range (95% control limits)			*0.01 - 0.69*		
Test item					
	422.75	2000	**0.35***	1.83	0[x]
	845.50	2000	**0.30**	1.83	0[x]
	1268.25	2000	**0.05**	1.66	14
	1691.00	2000	**0.20**	1.5	34
Positive control					
DC	0.0075	2000	**3.80 *** **	1.27	65
HCD[#] mean ± SD			4.31 ± 1.75		
Range (95% control limits)			*0.81 – 7.81*		

HCD: Historical control data (January 2019-April 2020; n = 40 for all conditions)

MMC: Mitomycin C; DC: Demecolcine; CP: Cyclophosphamide

[* and ***]: statistically significantly increased, χ^2 test, * $p < 0.05$, ***$p < 0.001$

[x]: Cytostasis was defined 0 when the relative CBPI value is equal to or higher than the solvent control

[a]: The percentage of micronucleated cells determined in a sample of 2000 binucleate cells (4000 for vehicle)

III. CONCLUSION

Under the conditions of the test and based on the experimental findings, glyphosate did not induce any toxicologically significant increases in the frequency of binucleate cells with micronuclei in either the absence or presence of a metabolising system. The test item was therefore considered to be non-clastogenic and non-aneugenic to human lymphocytes *in vitro*.

Assessment and conclusion by applicant:
Negative for induction of micronuclei in human peripheral lymphocytes *in vitro*, in the presence and absence of metabolic activation.

The study was conducted compliant with GLP and according to OECD guideline 487 (2016). There were no deviations. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate (batch AZM30320T0; purity 91.8%) was negative for the induction of micronuclei (induction of chromosome breaks and/or gain or loss) in human peripheral lymphocytes with and without metabolic activation under the conditions of this *in vitro* micronucleus assay.

B.6.4.2. In vivo studies in somatic cells

B.6.4.2.1. *In vivo studies in somatic cells – in vivo micronucleus, study 1*

Data point	CA 5.4.2/001 ***Remark RMS: This study was not included in the reference list. The applicant is requested to add this study to the reference list.***
Report author	[redacted]
Report year	2012
Report title	Glyphosate TGAI: Micronucleus test of glyphosate TGAI in mice.
Report No	485-1-06-4696
Document No	[redacted]-0112-6927-003
Guidelines followed in study	OECD 474 (1997), US EPA OPPTS 870.5395 (1998), EC 440/2008 B.12 (2008)
Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD TG 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study only 2000 polychromatic erythrocytes were evaluated, since that was required in the previous OECD guideline (1997). In addition, the percentage of polychromatic erythrocytes among total erythrocytes was determined for 200 erythrocytes instead of 500 erythrocytes. Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity observed, but dose levels included limit concentrations specified in the current guideline. In addition, the evaluation criteria specified in the study report did not consider historical control data of solvent controls. The deviations were not expected to significantly impact the study outcome. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Conclusion GRG: Valid, Category 2a Conclusion AGG: The study is considered to be acceptable.

Full summary

Glyphosate TGAI (TSN105914, batch: 20061109, purity: 98.9 %) was assessed for its genotoxic potential in a micronucleus test. Based on the results of a preliminary toxicity study, in which no systemic toxicity was observed at 2000 mg/kg bw/day, groups of six male mice were treated with a limit dose of 2000 mg/kg bw/day for two consecutive days. The test item was dissolved in vegetable oil and administered by oral gavage at a constant dosage volume of 10 mL/kg bw. In addition, 6 male mice were treated with the solvent under the same experimental conditions or received a single intraperitoneal injection of the positive control (1.0 mg/kg bw/day mitomycin C).

Mortality, clinical signs of toxicity and individual body weights were monitored. 24 hours following the last dose, the animals were sacrificed and bone marrow smears were prepared. For each animal, a minimum of 2000 polychromatic erythrocytes (PCE) was scored for the presence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was recorded from a minimum of 200 erythrocytes.

Treatment with 2000 mg/kg bw/day glyphosate TGAI did not induce any clinical signs of systemic toxicity and no changes in body weight. Furthermore, there was no statistically significant difference in the ratio of PCE/NCE, confirming that the test item did not induce bone marrow toxicity in the animals.
In addition, there was no statistically significant increase in the frequency of micronucleated PCE in glyphosate TGAI-treated mice when compared with the solvent control group.

Incidences of micronucleated PCE in the solvent and positive control groups were within the range of the laboratory's historical control data, demonstrating the capability of the test animals to respond to mutagenic substances and confirming the sensitivity of the test.

Based on the experimental findings and under the conditions of the test, glyphosate TGAI has no potential to induce micronuclei in the bone marrow of Swiss albino mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate TGAI

Identification:	TSN105914
Description:	White to off-white crystals
Lot/Batch #:	20061109
Purity:	98.9 %
Stability of test compound:	The stability of the test item at storage conditions was guaranteed until the expiry date 20 Apr 2014. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	Vegetable oil

2. Control materials

Solvent (vehicle) control:	Vegetable oil
Positive control:	Mitomycin C, 1 mg/kg bw/day

3. Test animals:

Species:	Mouse
Strain:	NMRI
Sex:	Males
Source:	[redacted]
Age at dosing:	8 - 9 weeks
Mean weight at dosing:	35 – 50 g
Acclimation period:	6 days
Diet/Food:	Teklad Certified Global 16 % Protein Rodent Diet, *ad libitum*
Water:	UV sterilized drinking water, filtered through a reverse osmosis water filtration system, *ad libitum*
Housing:	In groups of 6/cage polypropylene mouse cages measuring 29 x 37.5 x 14 cm with stainless steel grid top and clean rice husk bedding

4. Environmental conditions:

Temperature:	19 - 22 °C
Humidity:	64 – 65 %
Air changes:	Minimum 15/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	2000 mg/kg bw
Concentrations:	Not specified
Dose volume:	Not specified
Number of animals:	3/sex
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	2000 mg/kg bw/day
Concentrations:	200 mg/mL
Dose volume:	10 mL/kg bw
Number of animals:	6 males/group
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 28 May – 07 Aug 2012
Finalisation date: 13 Sep 2012

2. Animal assignment and treatment:

Preliminary toxicity study:
A dose range finding study was conducted to determine the maximum tolerated dose (MTD). Three male and three female mice were treated at the dose level of 2000 mg/kg bw/day for two consecutive days.
Mortality, clinical symptoms and change in body temperature were monitored up to 72 hours after the initial dose. The rectal temperature was measured prior to dosing, approx. 2, 5 and 24 hours after dosing and before sacrifice. Based on the results of the preliminary toxicity study, 2000 mg/kg bw/day were selected as dose level for the micronucleus test.

Main micronucleus test:
Groups of 6 male mice received a dose of 2000 mg/kg bw/day for two consecutive days. The test item was dissolved in vegetable oil and administered by oral gavage at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of mice were treated twice with the vehicle following the same route or received a single intraperitoneal injection of the positive control (1.0 mg/kg bw/day mitomycin C).
The animals were observed for clinical signs of toxicity post-dosing and pre-sacrifice. In addition, individual body weights were recorded prior to dosing and before sacrifice. The animals were sacrificed by CO_2 asphyxiation 24 hours after the second dose.

3. Slide preparation:

Femur bones from the sacrificed animals were excised and the epicondyle tips were removed. Bone marrow was flushed out with foetal bovine serum and the collected cells were pelleted by centrifugation. After re-suspension in 0.2 – 0.3 mL medium, the pellet was dissociated with a pipette and a drop of cell suspension was smeared on a clean slide and allowed to air-dry. For each animal two slides were prepared. The cells were fixed with absolute methanol and air-dried for 15 – 20 minutes, followed by 5 % Giemsa staining for 25 minutes. Subsequently, the slides were rinsed with distilled water, air-dried and mounted.

4. Slide evaluation:

Slides were randomly coded and evaluated by microscopical analysis. A minimum of 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was recorded from a minimum of 200 erythrocytes.

5. Statistics:

The percentage of micronucleated polychromatic erythrocytes (PCE) and the ratio of PCE to normochromatic erythrocytes (NCE) was statistically analysed using Bartlett's test and Analysis of Variance (ANOVA), followed by Dunnett's test to determine the level of significant differences between the vehicle control and the treatment group. Where data did not meet the homogeneity of variance, Student's t-test was performed

to determine the level of significant difference between the vehicle control, treatment group and the positive control group.

6. Acceptance criteria:

The study was considered valid if the following criteria were met:

- The prepared slides had uniform staining properties and a sufficient number of polychromatic erythrocytes (PCE) to allow accurate micronucleus determination.
- The solvent controls were in the range of the laboratories historical control data.
- The positive controls were in the range of the laboratories historical control data.
- At least 5 animals per group and sex were evaluated.
- The PCE to erythrocyte ratio was not less than 20 % of the solvent control.

7. Evaluation criteria:

A test substance was considered positive for induction of micronuclei when the following criteria were met:

- There was a dose-dependent, statistically significant increase in the incidence of micronuclei or increase in a single dose group.
- The result was of biological relevance.

A test item was considered negative for induction of micronuclei if no evident statistically significant increase in the numbers of micronucleated polychromatic erythrocytes (mPCE) was observed, relative to the concurrent and established historical control frequencies for PCE induction.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in this study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

There was no mortality and no adverse effects of systemic toxicity observed upon treatment with glyphosate TGAI. In addition, there was no impact on body temperature 24 and 48 hours after treatment.
Based on these findings, 2000 mg/kg bw/day were selected as the limit dose level for the micronucleus test.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:
No mortality occurred.

Clinical signs of toxicity:
Clinical signs of toxicity were not observed.

Body weight:
Body weights were comparable among all groups during the experimental period.

Evaluation of bone marrow slides:

Upon treatment with glyphosate TGAI, there was no statistically significant change in the ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE), indicating that there was no evidence of cell proliferation or bone marrow cytotoxicity.
In addition, there was no statistically significant increase in the frequency of micronucleated PCE (mPCE) in glyphosate TGAI-treated mice when compared with the solvent control group.
Incidences of mPCE in the solvent and positive control groups were within the range of the laboratory's historical control data, demonstrating the capability of the test animals to respond to mutagenic substances and confirming the sensitivity of the test.

Table B.6.4.2.1-1: Glyphosate TGAI: Micronucleus test of glyphosate TGAI in mice ([redacted], 2012), summary of genotoxicity data

Treatment	Dose (mg/kg bw/day)	Number of animals	Total number of PCE	Total number of mPCE	Mean mPCE	Mean% MNPCE	Mean PCE/NCE ratio
Vehicle	-	6	12014	4	0.667	0.033	0.525
HCD vehicle[#]							
mean ± SD						0.02 ± 0.02	0.58 ± 0.04
range						0.00 - 0.07	0.48 - 0.65
Test item	2000	6	12019	0	0.000	0.000	0.531
MMC	1.0	6	12030	300	50.000**	2.492**	0.687*
HCD positive control[#]							
mean ± SD						1.40 ± 0.36	0.57 ± 0.04
range						0.81 - 2.52	0.45 - 0.69

mPCE: micronucleated polychromatic erythrocytes

MMC: Mitomycin C, positive control

* and **: statistically significant at $p < 0.05$ and $p < 0.011$, respectively

[#] Historical control data, generated in the laboratory from Sep 2009 - Feb 2012 (data from male animals only)

III. CONCLUSION:

Based on the experimental findings glyphosate TGAI, administered at a limit dose of 2000 mg/kg bw/day, did not induce micronuclei in bone marrow in mice *in vivo*.

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male Swiss albino mice i*n vivo*.
The study was performed in compliance with GLP and according to OECD guideline 474 (1997). There were some deviations when compared to the current OECD guideline 474 (2016), which were considered to be of minor degree. The number of polychromatic erythrocytes investigated was 2000, corresponding to the number required by the previous guideline (1997). In addition, the percentage of polychromatic erythrocytes among total erythrocytes was determined for 200 erythrocytes instead of 500 erythrocytes. Evaluation criteria specified in the study report did not consider historical control data, but the values obtained in the present study were in line with historical controls. Bone marrow toxicity, indicated by a reduced polychromatic to normochromatic erythrocyte ratio or signs of systemic toxicity, was not evident, but the test was performed at limit dose levels in line with the current guideline. Therefore, the deviations were considered to not compromise the scientific validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS:
It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male Swiss albino mice i*n vivo* under the conditions of this test. The study is considered to be acceptable as the noted deviations are not considered to have an impact on the validity of the study.
However, it should be noted that bone marrow exposure was not shown in this study as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.

B.6.4.2.2. *In vivo studies in somatic cells – in vivo micronucleus, study 2*

Data point	CA 5.4.2/002 ***Remark RMS: reference was not included in the reference list. The applicant is requested to add this reference to the reference list.***
Report author	[redacted]
Report year	2012
Report title	Glyphosate Technical – Micronucleus Assay in Bone Marrow Cells of the Mouse.
Report No	1479200
Document No	Not reported
Guidelines followed in study	OECD 474 (1997), US EPA OPPTS 870.5395 (1998), EC 440/2008 B.12 (2008)
Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD TG 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study only 2000 polychromatic erythrocytes were evaluated, since that was required in the previous OECD guideline (1997). Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity observed, but dose levels included limit concentrations specified in the current guideline. In addition, the acceptance and evaluation criteria specified in the study report did not consider historical control data.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

Glyphosate technical (BX20070911, batch: 569753, purity: 96.3 %) was tested for its genotoxic potential in mice using the micronucleus test. Based on the results of a preliminary toxicity study, in which no toxicity was observed at 2000 mg/kg bw, groups of seven male mice each received a single oral dose of 2000 mg/kg bw. The test item was dissolved in 1 % (w/w) carboxymethyl cellulose and administered at a constant dosage volume of 20 mL/kg bw. Groups of 5 male control animals received the vehicle or the positive control (40 mg/kg bw cyclophosphamide in sterile water).

About 1, 2-4, 6, 24 and 48 hours after dosing, the animals were examined for signs of systemic toxicity. Bone marrow was sampled 24 and 48 hours after dosing and for each animal 2000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was determined per 2000 erythrocytes.

Treatment with glyphosate technical did not induce signs of systemic toxicity or mortality in the animals. Further, based on the ratio of PCE among the total number of erythrocytes, there was no evidence for bone marrow toxicity.

In addition, there was no biologically relevant and no statistically significant increase in the frequency of micronucleated PCE of test item-treated animals at any sampling time point when compared to vehicle controls.

Incidences of micronuclei in the solvent and positive control groups were within the range of the laboratory's historical control data, confirming the sensitivity of the test and demonstrating the capability of the test animals to respond to mutagenic substances.

Based on the experimental findings, the test item did not induce clastogenic/aneugenic effects in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

	Glyphosate Technical
Identification:	BX20070911
Description:	White solid
Lot/Batch #:	569753
Purity:	96.3 %
Stability of test compound:	The stability of the test item under storage conditions (< 30° C) was guaranteed until March 2015. The stability of the test item in solvent (vehicle) was not indicated by the sponsor.
Solvent (vehicle) used:	1 % carboxymethyl cellulose (CMC)

2. Control materials

Solvent (vehicle) control:	1 % carboxymethyl cellulose (CMC)
Positive control:	Cyclophosphamide, 40 mg/kg bw in sterile water

3. Test animals:

Species:	Mouse
Strain:	NMRI
Sex:	Males
Source:	[redacted]
Age at study initiation:	8 - 9 weeks
Mean weight at dosing:	35.5 ± 1.8 g (range: 32.4 – 38 g)
Acclimation period:	Minimum 5 days
Diet/Food:	Pelleted standard diet (Harlan laboratories B.V., Horst, The Netherlands), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in Macrolon type II/III cages with wire mesh top and granulated soft wood bedding

4. Environmental conditions:

Temperature:	22 ± 2 °C
Humidity:	45 – 65 %
Air changes:	Not specified
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	2000 mg/kg bw
Concentrations:	Not reported (calculated: 100 mg/mL)
Dose volume:	20 mL/kg bw
Number of animals:	2 males and 2 females
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	2000 mg/kg bw
Concentrations:	Not reported (calculated: 100 mg/mL)
Dose volume:	20 mL/kg bw
Number of animals:	7 males/group
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 04 – 25 Apr 2012
Finalisation date: 28 Sep 2012

2. Animal assignment and treatment:

Preliminary toxicity study:
The test item was dissolved in 1 % carboxymethyl cellulose (CMC) and administered by oral gavage at a constant dosage volume of 20 mL/kg bw to groups of 2 male and 2 female mice at a single dose of 2000 mg/kg bw.
The animals were examined for acute toxic symptoms about 1, 2-4, 6, 24, 30 and 48 hours after administration of the test substance. Since no signs of toxicity were evident at 2000 mg/kg bw, testing of lower doses was not regarded to be required.
Based on the results of the preliminary toxicity study, 2000 mg/kg bw were selected as dose level for the micronucleus test.

Main micronucleus test:
Groups of 7 male mice per dose level were administered a single dose of 2000 mg/kg bw by oral gavage at a constant dosage volume of 20 mL/kg bw. Similar constituted groups of 5 mice/dose level received the vehicle (1 % carboxymethyl cellulose) or the positive control (40 mg/kg bw cyclophosphamide).
Except for the positive control group, all mice were examined for acute toxic symptoms about 1, 2-4, 6, 24 and 48 hours after dosing. The animals were sacrificed 24 and 48 hours after test substance administration by CO_2 asphyxiation followed by bleeding.

3. Slide preparation:

After sacrifice the femurs of the animals were removed, the epiphysis were cut off and the marrow was flushed out with foetal calf serum. After centrifugation and re-suspension, a small drop of re-suspended cell pellet was spread on a slide. The smear was air-dried, stained with May-Grünwald/Giemsa and mounted with coverslips. At least one slide was made from each bone marrow sample.

4. Slide evaluation:

Slides were coded and evaluated by microscopical analysis at 100 x magnification. For each animal, 2000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was determined from the same slide and expressed in PCE per 2000 erythrocytes.

5. Statistics:

The results were evaluated using the non-parametric Mann-Whitney U-test.

6. Acceptance criteria:

The study was considered valid if the following criteria were met:

- At least 5 animals per group were evaluated.
- The PCE to erythrocyte ratio was not less than 20 % of the negative control.

- The positive control showed a statistically significant and biologically relevant increase of micronucleated PCE (mPCE) compared to the negative control.

7. Evaluation criteria:

The test item was considered mutagenic if the following criteria were met:

- It induced either a dose-related or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group.
- There was a biological relevance of the result.

A test item that failed to produce a biologically relevant increase in the number of micronucleated polychromatic erythrocytes was considered non-mutagenic in this system.
A test item failing to meet the criteria for a positive or negative response was judged equivocal in this assay and considered for further investigation.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in this study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

There were no signs of systemic toxicity observed. Based on these findings, 2000 mg/kg bw were selected as dose level for the micronucleus test.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:
No mortality occurred.

Clinical signs of toxicity:
Clinical signs of toxicity were not observed.

Evaluation of bone marrow slides:

Treatment with glyphosate technical did not affect the mean number of polychromatic erythrocytes (PCE) when compared to the mean value of PCE in the solvent control, indicating that the test item did not induce bone marrow toxicity.
In addition, there was no biologically relevant and no statistically significant increase in the frequency of micronucleated PCE of test item-treated animals at any sampling time point when compared to vehicle controls.
Incidences of micronuclei in the solvent and positive control groups were within the range of the laboratory's historical control data, confirming the sensitivity of the test and demonstrating the capability of the test animals to respond to mutagenic substances.

Table B.6.4.2.2-1: Micronucleus Assay in Bone Marrow Cells of the Mouse ([redacted] 2012), summary of genotoxicity data

Treatment	Dose (mg/kg bw)	Sampling time	mPCE ± SD /2000 PCE	% mPCE	PCE/2000 erythrocytes mean ± SD
DMSO	-	24 h	3.2 ± 3.6	0.160	1245
	-	48 h	1.4 ± 1.1	0.070	1197
HCD solvent control[#]					
mean ± SD(%)				0.108 ± 0.039	
range			0 - 9	0.010 - 0.250	
Test item					
	2000	24 h	2.3 ± 0.5	0.114	1247
	2000	48 h	1.1 ± 1.3	0.057	1092

Table B.6.4.2.2-1: Micronucleus Assay in Bone Marrow Cells of the Mouse ([redacted], 2012), summary of genotoxicity data

Treatment	Dose (mg/kg bw)	Sampling time	mPCE ± SD /2000 PCE	% mPCE	PCE/2000 erythrocytes mean ± SD
Positive control					
CPA	40	24 h	40.2 ± 18.2	2.010	1149
HCD positive control[#]					
mean ± SD(%)				2.533 ± 0.632	
range			8 - 139	0.858 - 4.370	

mPCE: micronucleated polychromatic erythrocytes

CPA: cyclophosphamide

HCD[#]: Historical control data from 2006 - 2011

III. CONCLUSION:

Based on the experimental findings glyphosate technical did not induce micronuclei in bone marrow in mice and is therefore considered negative for clastogenicity/aneuploidy *in vivo*.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male NMRI mice *in vivo*.

The study was performed under GLP conditions and in accordance with OECD guideline 474 (1997). There were only minor deviations when compared to the current OECD 474 (2016). The number of polychromatic erythrocytes investigated was 2000, corresponding to the number required by the previous guideline (1997). In addition, the evaluation criteria specified in the study report did not consider historical control data, but the values obtained in the present study were in line with historical controls. Bone marrow toxicity, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, or signs of systemic toxicity were not evident, but the test was performed at limit dose levels in line with the current guideline. The deviations were considered to not compromise the validity of the study. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male NMRI mice i*n vivo* under the conditions of this test. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
The study is considered to be acceptable as the noted deviations are not considered to have an impact on the study validity.

B.6.4.2.3. *In vivo studies in somatic cells – in vivo micronucleus, study 3/study 4 (same study)*

Data point	CA 5.4.2/003
Report author	[redacted]
Report year	2008
Report title	Evaluation of the mutagenic potential of Glyphosate Technical by Micronucleus assay in mice
Report No	[redacted]-3996.402.395.07
Document No	Not reported
Guidelines followed in study	OECD 474 (1997)

Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study, 2000 polychromatic erythrocytes were evaluated (requirement of previous OECD guideline (1997)). In addition, the test item was administered via intraperitoneal injection, which is not representative for a human route of exposure. Historical control data on the mean number of micronucleated polychromatic erythrocytes / 2000 polychromatic erythrocytes were reported, but no standard deviations were given. Acceptance criteria were not reported and evaluation criteria specified in the study report were inconsistent with the historical control data provided.
Previous evaluation	Not accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Refer to B.6.4.2.4

B.6.4.2.4. *In vivo studies in somatic cells – in vivo micronucleus, study 3/study 4 (same study)*

Data point	CA 5.4.2/004
Report author	[redacted]
Report year	2010
Report title	First amendment to the report: Evaluation of the mutagenic potential of Glyphosate Technical by Micronucleus assay in mice
Report No	[redacted]-3996.402.395.07
Document No	Not reported
Guidelines followed in study	OECD 474 (1997)
Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study, 2000 polychromatic erythrocytes were evaluated (requirement of previous OECD guideline (1997)). In addition, the test item was administered via intraperitoneal injection, which is not representative for a human route of exposure. Historical control data on the mean number of micronucleated polychromatic erythrocytes / 2000 polychromatic erythrocytes were reported, but no standard deviations were given. In addition, evaluation criteria specified in the study report were inconsistent with the historical control data provided.
Previous evaluation	Not accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 3a **Conclusion AGG:** The study is considered as not acceptable (refer to RMS conclusion and comments below).

Glyphosate technical (batch: 20070606, purity 98.0 %) was tested for its genotoxic potential in a micronucleus test conducted in male and female Swiss mice (main study and amendment).

Based on the results of a preliminary toxicity study, in which mortality was observed at ≥ 500 mg/kg bw/day and bone marrow toxicity was evident at ≥ 250 mg/kg bw, dose levels for the main micronucleus test were selected. 5 mice per sex and dose received on two consecutive days (24 hours apart) doses of 15.62, 31.25, 62.5, 125, 250 and 375 mg/kg bw/day by intraperitoneal injection. Similar constituted groups of mice received the vehicle (corn oil) or the positive control (25 mg/kg bw/day cyclophosphamide). Bone marrow sampling was performed 24 hours after the second dose administration, followed by preparation and staining of smears. For each animal, 2000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei. At the same time, normochromatic erythrocytes (NCE) with micronuclei were recorded. The ratios of PCE/NCE were determined among the first 2000 PCE.

Intraperitoneal injection of glyphosate technical was associated with clinical signs of toxicity in males and females at ≥ 125 mg/kg bw/day. Common signs of toxicity were bristling and tachypnea, which were observed in both sexes at 125, 250 and 375 mg/kg bw/day throughout the whole study period. After the second dose, lethargy was observed in the animals, which was reversible in the 125 mg/kg bw/day dose group but remained until study termination in the

animals of the 250 and 375 mg/kg bw/day dose groups. The animals of the high dose group (375 mg/kg bw/day) further showed loss of motor coordination 30 minutes after the second dose, which was reversible up to 4 hours post dosing. These signs of systemic toxicity were clearly attributed to treatment with glyphosate technical and considered to be of toxicological relevance.

A statistically significant increase in the ratio of PCE/NCE when compared to solvent control animals was observed at ≥ 250 mg/kg bw/day in males and at 375 mg/kg bw/day in females. The observation indicated that strong bone marrow toxicity was evident in the animals.
There was no statistically significant increase in the incidence of micronucleated PCE (mPCE) when compared to solvent control animals, up to the highest dose tested.

The frequency of mPCE of solvent and positive control animals were within the range of the laboratories historical control data, demonstrating the validity and the sensitivity of the test.

Based on the experimental findings and under the conditions of the test, glyphosate technical did not induce micronuclei in bone marrow in male and female mice and was therefore considered negative for clastogenicity/aneugenicity *in vivo*.

M. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate Technical

Identification:	AGR-0790/07
Description:	Not specified
Lot/Batch #:	20070606
Purity:	98.0 %
Stability of test compound:	As given in the study report, the substance was described[8] to not photochemically degrade in buffered water and to be stable in air. It was further stated to be stable to hydrolysis at pH 3, 6 and 9 at 5 – 35 °C.
Solvent (vehicle) used:	Corn oil

2. Control materials

Solvent (vehicle) control:	Corn oil
Positive control:	Cyclophosphamide monohydrate, 25 mg/kg bw/day in physiological solution

3. Test animals:

Species:	Mouse
Strain:	Swiss albino
Sex:	Male and female
Source:	[redacted]
Age at study initiation:	7 - 12 weeks
Mean weight at dosing:	Approximately 30 g
Acclimation period:	5 days
Diet/Food:	Purina Labina (Purina do Brasil Ltda.), *ad libitum*
Water:	*ad libitum*
Housing:	5/sex in polypropylene cages closed with a metallic grid and padded out with sterile sawdust bedding.

[8] Tomlin, C.D.S. (2006-2007): The e-Pesticide Manual (14th Edition) Version 4.0; Software engineered by P.J. Mann – Web Design & Consultancy. BCPC (British Crop Protection Council) ISBN 1 901396 42 8

4. Environmental conditions:

Temperature:	20 - 24 °C
Humidity:	50 - 70 %
Air changes:	10 – 15/h
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	62.5, 125, 250, 500 and 1000 mg/kg bw/day
Concentrations:	Not specified
Dose volume:	Not specified
Number of animals:	3/sex/group
Route of administration:	Intraperitoneal injection

b) Main micronucleus test

Dose levels:	15.62, 31.25, 62.5, 125*, 250* and 375* mg/kg bw/day [9*]
Concentrations:	1.04, 2.08, 4.16, 8.33*, 16.66*, 25.00* mg/mL
Dose volume:	15 mL/kg bw
Number of animals:	5/sex/group
Route of administration:	Intraperitoneal injection

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 29 May – 13 Aug 2008
Finalisation date: 29 Sep 2008 (study report) and 31 Aug 2010 (first amendment to study report)

2. Animal assignment and treatment:

Preliminary toxicity study:
The test item was dissolved in corn oil and administered via intraperitoneal injection to groups of six mice per dose level (3/sex/group). The animals received two injections 24 hours apart at doses of 62.5, 125, 500 and 1000 mg/kg bw/day. Following administration, the animals were observed for signs of mortality. Surviving animals were sacrificed 24 hours following the second dose administration.

Bone marrow toxicity was assessed in surviving animals by determining the ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) among 200 PCE per animal.
Based on the results of the preliminary toxicity study, the dose levels for the main micronucleus test were chosen. In the original study report, 62.5 mg/kg bw/day was selected as highest dose level. In the first amendment to the study report, 3 higher dose levels were tested with a maximum dose level of 375 mg/kg bw/day.

Main micronucleus test:
Groups of 5 mice/sex received two separate intraperitoneal injections of 15.62, 31.25, 62.5, 125*, 250* and 375* mg/kg bw/day 24 hours apart[9]. Similar constituted groups received the vehicle (corn oil) or positive control (25 mg/kg bw/day cyclophosphamide in physiological solution).
About 24 hours after the second injection, the animals were sacrificed by CO_2 asphyxiation and bone marrow slides were prepared from each animal.

3. Slide preparation:

After sacrifice, the femurs of the animals were excised, dissected and the marrow was flushed out with fetal calf serum. After centrifugation and re-suspension in fetal calf serum, a drop of re-suspended cells was smeared on microscope slides. Two slides were prepared for each animal. The smears were air-dried, fixed in 70 % ethanol

* tested in amendment to study report.

for 10 minutes, stained with Wright's concentrated solution for 3 minutes, placed in Wright's phosphate buffer solution and rinsed. Thereafter, the slides were immersed for 10 minutes in Giemsa buffer – deionised water solution, washed in streamed water, dried and assembled in Permount.

4. Slide evaluation:

Slides were coded and evaluated by microscopical analysis at 1000 x magnification. For each animal, 2000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei. At the same time, normochromatic erythrocytes (NCE) were screened for micronuclei. The ratios of PCE/NCE were determined among the first 2000 PCE.

5. Statistics:

Differences in the incidence of micronucleated polychromatic (PCE) and normochromatic erythrocytes (NCE) per 2000 cells and the relation of PCE/NCE were compared using the Mann-Whitney U-test and the K'test for independent variable in accordance with Kruskal & Wallis Test.

6. Acceptance criteria:

Acceptance criteria were not specified in the study report.

7. Evaluation criteria:

The test item was considered positive for induction of micronuclei if the following criteria were met:

- There was a statistically significant, dose-related increase in the number of micronucleated polychromatic erythrocytes when compared to the solvent control.
- There was a reproducible and statistically significant positive response for at least one of the dose levels tested.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed. The substance was described to not photochemically degrade in buffered water and to be stable in air. It was further stated to be stable to hydrolysis at pH 3, 6 and 9 at 5 – 35 °C.

B. PRELIMINARY TOXICITY STUDY

Mortality was observed at 500 mg/kg bw/day in 1/3 males and 2/3 females and at 1000 mg/kg bw/day in 3/3 males and 3/3 females.

Bone marrow cytotoxicity, evident as a ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) lower than 20 % when compared to solvent control animals, was observed at 250 and 500 mg/kg bw/day.

Based on the results of the preliminary toxicity study, the dose levels for the main micronucleus assay were selected. In the original study report, 62.5 mg/kg bw/day was chosen as highest dose level. In the first amendment to the study report, 3 higher dose levels were included with a maximum dose level of 375 mg/kg bw/day.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

Clinical signs of toxicity were observed at 125, 250 and 375 mg/kg bw/day.

At 125 mg/kg bw/day, bristling was observed in 5/5 males and 5/5 females and tachypnea in 1/5 males and 1/5 females starting 30 minutes after the first dose. After the second dose, all males and females of this dose group showed bristling, tachypnea and lethargy starting 30 minutes post administration. Bristling and tachypnea were observed in all animals until study termination.

At 250 mg/kg bw/day, bristling and tachypnea was observed in 5/5 males and 5/5 females after the first and second dose until study termination. About 30 minutes after the second dose, all animals showed lethargy, which was still present in 1/5 males 4 hours after dosing and remained in 2/5 females until study termination.

At 375 mg/kg bw/day, 5/5 males and 5/5 females showed bristling and tachypnea immediately following test item administration (first and second dosing), which was observed in all animals until bone marrow sampling. 24 hours after the first dose, 5/5 males and 2/5 females showed agitation. After the second dose, 5/5 males and 5/5 females showed lethargy and loss of motor coordination within the first 30 minutes post dosing. The animals recovered from the loss of motor coordination within 4 hours post dosing, while lethargy was observed in 4/5 males and 3/5 females until study termination.

The signs of systemic toxicity were attributed to treatment and considered to be of toxicological relevance.

Evaluation of bone marrow slides:
Treatment with glyphosate technical at concentrations of 250 mg/kg bw/day and above caused a statistically significant change in the PCE/NCE ratio, indicating that bone marrow toxicity was evident at these concentrations. Bone marrow toxicity was observed in males at ≥ 250 mg/kg bw/day and in females at 375 mg/kg bw/day.

There was no statistically significant increase in the incidence of mPCE when compared to solvent control animals up to the highest dose tested.

The frequency of mPCE of solvent and positive control animals were within the range of the laboratories historical control data, demonstrating the validity and the sensitivity of the test.

Table 6.4.2.4-1: Evaluation of the mutagenic potential of Glyphosate Technical Micronucleus assay in mice ([redacted], 2008), summary of genotoxicity data for males and females

Treatment	Dose (mg/kg bw/day)	Sampling time	Males		Females	
			mPCE/2000 PCE mean ± SD	PCE/(PCE + NCE) mean	mPCE/2000 PCE mean ± SD	PCE/(PCE + NCE) mean
Corn oil	15 mL/kg bw[(a)]	24 h	0.0 ± 0.0	1.781	0.0 ± 0.0	1.772
	15 mL/kg bw[(b)]	24 h	0.4 ± 0.89	0.5147	0.4 ± 0.55	0.523
	HCD[#] mean	24 h	0.87	1.43 ± 0.72[$]	0.76	1.54 ± 0.68[$]
	HCD[##] mean	24 h	0.51	0.54 ± 0.08[$]	0.41	0.60 ± 0.36[$]
Test item	15.62[(a)]	24 h	0.0 ± 0.0	1.768	0.0 ± 0.0	1.791
	31.25[(a)]	24 h	0.2 ± 0.45	1.744	0.0 ± 0.0	1.761
	62.5[(a)]	24 h	0.6 ± 1.34	1.711	0.0 ± 0.0	1.787
	125[(b)]	24 h	0.2 ± 0.45	0.527	0.0 ± 0.0	0.528
	250[(b)]	24 h	0.0 ± 0.0	0.534*	0.0 ± 0.0	0.528
	375[(b)]	24 h	0.2 ± 0.45	0.556**	0.0 ± 0.0	0.555*
CPA	25[(a)]	24 h	23.0 ± 8.94**	1.549	12.2 ± 7.92**	1.728
	25[(b)]	24 h	8.0 ± 2.12**	0.535*	6.4 ± 1.67**	0.539*
	HCD[#] mean	24 h	9.30	1.31 ± 0.69[$]	9.20	1.35 ± 0.65[$]
	HCD[##] mean	24 h	10.02	0.59 ± 0.34[$]	9.45	0.54 ± 0.06[$]

mPCE: micronucleated polychromatic erythrocytes

CPA: cyclophosphamide, positive control

[$]: ± average amplitude

[a]: data from study report (2008), standard deviations were not included in study report but calculated from raw data

[b]: data from amendment to study report (2010)

HCD[#]: historical control data from the testing laboratory generated in Jan - Dec 2007

HCD[##]: historical control data from the testing laboratory generated in Jan - Dec 2009

Table 6.4.2.4-1: Evaluation of the mutagenic potential of Glyphosate Technical Micronucleus assay in mice ([redacted], 2008), summary of genotoxicity data for males and females

Treatment	Dose (mg/kg bw/day)	Sampling time	Males		Females	
			mPCE/2000 PCE mean ± SD	PCE/(PCE + NCE) mean	mPCE/2000 PCE mean ± SD	PCE/(PCE + NCE) mean

* $p \leq 0.05$ and ** $p \leq 0.01$ difference statistically significant from solvent control by Mann-Whitney (Kruskal Wallis) test

Table B.6.4.2.4-26.4.2: Evaluation of the mutagenic potential of Glyphosate Technical Micronucleus assay in mice ([redacted], 2008), summary of genotoxicity data for males and females combined

Treatment	Dose (mg/kg bw/day)	Sampling time	Both sexes combined	
			mPCE/2000 PCE mean ± SD	PCE/(PCE + NCE) mean
Corn oil	15 mL/kg bw[(a)]	24 h	0.0 ± 0.0	1.777
	15 mL/kg bw[(b)]	24 h	0.4 ± 0.70	0.519
Test item	15.62[(a)]	24 h	0.0 ± 0.0	1.780
	31.25[(a)]	24 h	0.1 ± 0.32	1.752
	62.5[(a)]	24 h	0.3 ± 0.95	1.749
	125[(b)]	24 h	0.1 ± 0.32	0.527
	250[(b)]	24 h	0.0 ± 0.0	0.531*
	375[(b)]	24 h	0.1 ± 0.32	0.556**
CPA	25[(a)]	24 h	17.6 ± 9.79**	1.639
	25[(b)]	24 h	7.2 ± 1.99**	0.537**

mPCE: micronucleated polychromatic erythrocytes

CPA: cyclophosphamide, positive control

[$]: ± average amplitude

[a]: data from study report (2008), standard deviations were not included in study report but calculated from raw data

[b]: data from amendment to study report (2010)

HCD[#]: historical control data from the testing laboratory generated in Jan - Dec 2007

HCD[##]: historical control data from the testing laboratory generated in Jan - Dec 2009

* $p \leq 0.05$ and ** $p \leq 0.01$ difference statistically significant from solvent control by Mann-Whitney (Kruskal Wallis) test

III. CONCLUSION

Based on the experimental findings and under the conditions of the test, glyphosate technical did not induce micronuclei in bone marrow in male and female mice *in vivo*.

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male and female Swiss albino mice i*n vivo*.

The study was performed under GLP conditions and according to OECD guideline 474 (1997). There were only minor deviations when compared to the current OECD guideline 474 (2016). The number of polychromatic erythrocytes investigated per animal was 2000 (instead of 4000), which was the number recommended by the

previous OECD guideline (1997). In addition, the test item was administered via intraperitoneal injection, which does not represent a typical human route of exposure. There was clear evidence of systemic toxicity and bone marrow toxicity in animals of both sexes, but there was no evidence for induction of micronuclei at any dose level.

The study is considered to provide supporting information, because two different batches of the test material were used in the original study and in the amendment to the study. In addition, the treatment of the animals used in the amendment was not described and the amendment does not replace a full study report. However, it can be assumed that the treatment was the same as in the original study.

Assessment and conclusion by RMS: The RMS is of the opinion that the study is considered not acceptable due to the assumptions with regard to the treatment and the deviations mentioned. This is in line with the previous assessment of the study. However, the results of the study showed that glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male and female Swiss albino mice i*n vivo* under the conditions of this test.

In the previous assessment the following was concluded by the RMS (RAR, 2015):
…. "In the original report, dose selection for the "definitive test" was justified with the outcome of a preliminary test. In this range finding experiment, 3 males and 3 females per dose level received i.p. glyphosate doses of 62.5, 125, 250, 500, or 1000 mg/kg bw. The top dose level resulted in 100 % mortality and at the next lower dose level of 500 mg/kg bw, one male and two female mice died. Based on a clear decrease in the PCE/NCE ratios in both sexes, the intermediate dose of 250 mg/kg bw was found to be cytotoxic. It was recommended that 125 mg/kg bw was the most appropriate high dose to be employed in the definitive test but, without further justification, 62.5 mg/kg bw was actually the highest dose used. That was much lower than in other studies in which the i.p. route had been also chosen ([redacted], 1999, ASB2012-11482; [redacted], 2006, ASB2012-11478).
In 2014, an amendment to this study was submitted ([redacted], 2010, ASB2014-9284). In this document, some results of testing glyphosate at dose level of 125, 250, and 375 mg/kg bw are reported. Clinical signs but no mortality were seen at all dose levels. It is not clear in which way this data is linked to the preliminary test that was performed as part of the original study since the dose levels were not exactly the same and the number of animals was different (this time 5 per sex and dose). Furthermore, in the amendment, more data on micronucleus incidences and PCE/NCE ratios at the dose levels of 15.62, 31.25, and 62.5 mg/kg bw was given, apparently based on 10 animals per sex and dose. It was confirmed that there was no clastogenic potential of the test substance. However, treatment of these animals was simply not described in the original report and the amendment cannot be considered a full study report. Taking all these deficiencies and uncertainties in the amendment as well as the use of only very low dose levels into account, assessment of the study as "not acceptable" by the RMS is maintained."

B.6.4.2.5. *In vivo studies in somatic cells – in vivo micronucleus, study 5*

Data point	CA 5.4.2/005
Report author	[redacted]
Report year	2008
Report title	Glyphosate Technical – Micronucleus Assay in Bone Marrow Cells of the Mouse
Report No	1158500
Document No	Not reported
Guidelines followed in study	OECD 474 (1997), EPA OPPTS 870.5395 (1998), 2000/32/EEC B.12 (2000)
Deviations from current test guideline OECD 474 (2016)	Only 2000 polychromatic erythrocytes were evaluated per animal instead of 4000 polychromatic erythrocytes as recommended by OECD guideline 474 (2016). Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity observed, but dose levels included limit concentrations specified in the current guideline. Acceptance criteria were not specified in the study report. In addition, historical controls were not considered in the evaluation criteria. The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)

GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable.

Glyphosate technical (batch: 20070545, purity: 99.1 %) was tested for its genotoxic potential in mice using the micronucleus test. Based on the results of a preliminary toxicity study, groups of six mice each received a single oral dose of 500, 1000 and 2000 mg/kg bw glyphosate technical. The test item was dissolved in 0.5 % (w/w) carboxymethyl cellulose and administered at a constant dosage volume of 20 mL/kg bw. Similar constituted groups of control animals received the vehicle (0.5 % carboxymethyl cellulose) or the positive control (40 mg/kg bw cyclophosphamide in deionised water). All animals were observed for clinical signs of toxicity at regular intervals. 24 and 48 hours after administration, the animals were sacrificed and bone marrow smears were prepared. For the incidence of micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored. To describe a cytotoxic effect, the ratio between PCE and normochromatic erythrocytes (NCE) was determined and expressed in PCE per 2000 erythrocytes.

Oral administration of glyphosate technical did not induce any clinical signs of toxicity or mortality in the animals. In addition, there was no statistically significant decrease in the ratio of PCE to the total amount of erythrocytes in any dose group or at any preparation interval, indicating that glyphosate technical did not induce cytotoxicity in the bone marrow.

A biologically relevant or statistically significant increase in the frequency of micronucleated polychromatic erythrocytes was not observed for the test item at any preparation interval and any dose level.

The positive control cyclophosphamide showed a marked increase in the frequency of induced micronuclei, demonstrating the sensitivity of the test system.
Based on the results of the test and under the experimental conditions chosen, the test item was considered to be negative for clastogenicity/aneugenicity in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate Technical

Identification:	S846011
Description:	White solid
Lot/Batch #:	20070545
Purity:	99.1 % (w/w)
Stability of test compound:	The stability of the test item at storage conditions (< 10° C) was guaranteed until the expiry date Oct 2009. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	0.5 % carboxymethyl cellulose (CMC)

2. Control materials

Solvent (vehicle) control:	0.5 % carboxymethyl cellulose (CMC), 20 mL/kg bw
Positive control:	Cyclophosphamide, 40 mg/kg bw in deionised water, 10 mL/kg bw

3. Test animals:

Species:	Mouse
Strain:	NMRI

Sex:	Males
Source:	[redacted]
Age at study initiation:	7 - 8 weeks
Mean weight at dosing:	39.0 ± 2.6 g
Acclimation period:	Minimum 5 days
Diet/Food:	Pelleted standard diet (Harlan Winkelmann GmbH, Borchen, Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually, in Makrolon type I cages with wire mesh top

4. Environmental conditions:

Temperature:	22 ± 3 °C
Humidity:	30 - 70 %
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	2000 mg/kg bw
Dose volume:	20 mL/kg bw
Number of animals:	2 males and 2 females
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	500, 1000 and 2000 mg/kg bw
Dose volume:	20 mL/kg bw
Number of animals:	500 and 1000 mg/kg bw: 6 males 2000 mg/kg bw: 12 males
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 25 Feb – 13 Mar 2008
Finalisation date: 09 Jun 2008

2. Animal assignment and treatment:

Preliminary toxicity study:
A preliminary study on acute oral toxicity was performed in male and female mice. Two animals per sex received a single dose of 2000 mg/kg bw by oral gavage. Formulations were applied at a constant dosage volume of 20 mL/kg bw. The animals were examined for acute toxic symptoms at intervals of around 1 hour, 2-4 hours, 6 hours, 24, 30 and 48 hours after administration of the test item.

Main micronucleus test:
Based on the results of the preliminary toxicity study, groups of six mice each received a single oral dose of 500, 1000 and 2000 mg/kg bw glyphosate technical, which was administered at a constant dosage volume of 20 mL/kg bw. Similar constituted groups of control animals received the vehicle (0.5 % carboxymethyl cellulose) or the positive control (40 mg/kg bw cyclophosphamide in deionised water). All animals were observed for acute toxic symptoms at intervals of around 1 hour, 2-4 hours, 6 hours (except for high dose group animals), 24 and 48 hours after test item administration.

Bone marrow sampling was performed 24 and 48 hours after treatment; the latter sampling time point was performed for the control and high dose group only. The animals were sacrificed by CO_2 asphyxiation followed by bleeding.

1. Slide preparation:

After sacrifice the femurs of the animals were removed, dissected and the marrow was flushed out with foetal calf serum. After centrifugation and re-suspension, a small drop of re-suspended cell pellet was spread on a glass slide. The smear was air-dried, stained with May-Grünwald/Giemsa and mounted with coverslips. At least one slide was made from each bone marrow sample.

2. Slide evaluation:

Slides were coded and examined using a NIKON microscope with 100x oil immersion objectives. For the incidence of micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored. To evaluate a potential cytotoxic effect, the ratio between PCE and normochromatic erythrocytes (NCE) was determined and expressed in PCE per 2000 erythrocytes.

The presence of micronuclei was scored for 5 males per group. The remaining 6th animal in the respective test group was kept as backup in case of spontaneous death.

3. Statistics:

All data were statistically analysed using the nonparametric Mann-Whitney U-test.

4. Acceptance criteria:

The test was considered valid when the following criteria were met:

- The negative controls were in the range of the laboratory's historical control data.
- The positive controls were in the range of the laboratory's historical control data.
- At least 4 animals per group were evaluated.
- The PCE to erythrocyte ratio was not less than 20 % to those of the negative control value.

5. Evaluation criteria:

The test substance was considered to be mutagenic if it induced either a statistically significant, dose-related increase or a clear statistically significant increase in the number of micronucleated PCE in a single dose group.

A test item was considered to be non-clastogenic if it failed to produce a biologically relevant increase in the number of micronucleated PCE.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations have not been performed in this study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

Animals treated at the limit dose of 2000 mg/kg bw did not express any toxic reactions. Based on the results of the preliminary toxicity study, 2000 mg/kg bw was selected as high dose level for the main micronucleus test. Since gender-specific differences in the sensitivity towards glyphosate technical were not observed, the main experiment was performed using only males.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

There were no clinical signs of toxicity observed.

Evaluation of bone marrow slides:

There was no decrease in the mean number of polychromatic erythrocytes among total erythrocytes in test item treated groups when compared to vehicle controls, indicating that glyphosate technical did not induce cytotoxicity in the bone marrow.

In addition, there was no biologically relevant or statistically significant increase in the frequency of micronucleated polychromatic erythrocytes at any preparation interval and any dose level.

A dose of the positive substance cyclophosphamide showed a marked increase in the frequency of induced micronuclei, demonstrating the capability of the test animals to respond to mutagens.

Table B.6.4.2.5-1: Micronucleus Assay in Bone Marrow Cells of the Mouse ([redacted], 2006) summary of genotoxicity data

Treatment	Dose (mg/kg bw)	Sampling time	mPCE ± SD /2000 PCE	% cells with micronuclei	PCE/2000 erythrocytes (mean ± SD)
Saline	20 mL/kg	24 h	1.40 ± 1.34	0.07	1202 ± 144
	20 mL/kg	48 h	1.40 ± 1.14	0.07	1153 ± 40
HCD vehicle[#] mean ± SD (%)				0.084 ± 0.031	
range				0.01 - 0.18	
Test item	500	24 h	1.60 ± 0.55	0.08	1147 ± 70
	1000	24 h	1.60 ± 0.55	0.08	1162 ± 186
	2000	24 h	1.40 ± 0.89	0.07	1173 ± 23
	2000	48 h	1.60 ± 1.14	0.08	1188 ± 78
CPA	40	24 h	63.0 ± 17.78	3.15	1030 ± 76
HCD positive control[#] mean ± SD (%)				1.973 ± 0.630	
range				0.77 – 3.69	

mPCE: micronucleated polychromatic erythrocytes

CPA: cyclophosphamide, positive control

[#] HCD Historical control data from the laboratory generated in 2002 - 2007 (vehicle control: males and females combined based on 293 experiments in males and 275 experiments in females; positive control: males and females combined based on 292 experiments in males and 274 experiments in females)

III. CONCLUSION

Based on the results of the present study, glyphosate technical did not induce micronuclei in bone marrow of male mice *in vivo*. Thus, the test item was considered to be negative for clastogenicity/aneugenicity under the conditions of the test.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male mice *in vivo*.
The study was performed under GLP conditions and in accordance with OECD guideline 474 (1997). There were only minor deviations when compared to the current OECD 474 (2016), which were considered to not compromise the validity of the study. The number of polychromatic erythrocytes investigated was 2000, corresponding to the number required by the previous guideline (1997). Bone marrow toxicity, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, or systemic toxicity were not observed. However, the test was performed at limit dose levels in line with the current guideline, therefore the deviation is considered negligible. The study is considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male mice i*n vivo* under the conditions of this test. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
The study is considered acceptable as the noted deviations are not considered to have an impact on the study validity.

B.6.4.2.6. *In vivo studies in somatic cells – in vivo micronucleus, study 6*

Data point:	CA 5.4.2/006
Report author	[redacted]
Report year	2007
Report title	Mammalian Erythrocyte Micronucleus Test for GLIFOSATO TÈCHNICO HELM
Report No	RL33393/2007-3.0MN-B
Document No	Not reported
Guidelines followed in study	OECD 474 (1997), Commission Directive 2000/32/EC B.12
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate technical (batch: 2007091801, purity: 98.01 %) was assessed for genotoxic effects in a micronucleus test in male Swiss mice *in vivo*. The dose levels for the micronucleus assay were selected based on the results of a preliminary toxicity study, in which 30 mg/kg bw/day turned out to be the maximum tolerated dose. Groups of six mice received on two consecutive days doses of 8.0, 15.0 and 30.0 mg/kg bw/day by oral gavage. Concurrent control animals received the vehicle (deionized water) or 75 mg/kg bw/day of the well-known mutagen cyclophosphamide (positive control). Bone marrow sampling was performed 24 hours after administration of the second dose. Smears were prepared from the femoral bone marrow of each animal and 3000 polychromatic erythrocytes (PCE) were scored for the presence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was determined for each animal by counting a total of 2000 erythrocytes.
Short description of results:	Oral treatment with glyphosate technical did not induce any mortality in the animals and signs of systemic toxicity were as well not reported. In addition, the ratio of PCE to NCE was not affected upon treatment with the test item up to the highest dose level. When compared to solvent control animals, treatment with glyphosate doses of 8.0 and 15.0 mg/kg bw/day did not lead to a statistically significant increase the frequency of micronucleated PCE. At 30 mg/kg bw/day, a statistically significant increase in micronucleated PCE was observed, but the values remained within the range of historical control data and were therefore considered to be without biological relevance. Micronuclei formation in the solvent controls was consistent to the historical control data. The positive control cyclophosphamide showed the expected result and demonstrated the functionality of the test system. Under the conditions of the test, glyphosate technical was considered negative for clastogenic/aneugenic activity in male mice *in vivo*.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study is considered to be not acceptable since the dose levels were far too low for any meaningful conclusion with regard to micronucleus formation. In the original report, some justification for dose selection is given, based on a range-finding test suggesting effects at even lower dose levels. These findings were obviously in contradiction to more reliable acute toxicity tests with glyphosate in the mouse. In other

	micronucleus assays described in this section, much higher dose levels were assessed. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as described by GRG. The following information is copied from the previous CLH report (BAuA, 2016), ″ a third study had caused some discussion during the ongoing evaluation process of glyphosate in the EU, in particular during the public consultation in 2014, since a "positive" result has been claimed. For consistency, this study is briefly reported here. [redacted] (2007, CA 5.4.2/006, considered as invalid in the MCA document) administered 98% pure glyphosate from a Brazilian manufacturer to male Swiss mice (six per dose level). The animals were dosed twice with a 24-hour interval between by oral gavage. Sampling took place 24 hours after the second dose. The dose levels were 8, 15, and 30 mg/kg bw, based on toxicity observed in a range - finding test. On bone marrow slides, 3000 PCE per animal were scored for micronuclei. At the highest dose level, there was a statistically significant increase in micronucleus frequency (Chi- square test, p = 0.02). Against the large database that is available for glyphosate, this finding is surprising, as well as the high toxicity. In the range finding experiment, two animals that had been administered 2000 mg/kg bw died on day 3 after having shown ataxia and prostration before. The same observations were made in 3 animals which received an oral dose of 320 mg/kg bw. They all died on day 2. Even at a dose level of 50 mg/kg bw, one out of three treated animals died on day 1. The occurrence of deaths and clinical signs at relatively low dose levels was obviously in contradiction to the available acute toxicity tests with glyphosate in mice ([redacted], 1995, ASB2012-11382 (CA 5.2.1/016); [redacted], 1991, TOX9551089 (CA 5.2.1/027); [redacted] 1991 (CA 5.2.1/025), TOX9552320; [redacted], 1994, TOX9551624, (CA 5.2.1/023)) revealing an LD_{50} higher than 2000 or even 5000 mg/kg bw. In line with that, much higher dose levels were employed in the other (negative) micronucleus assays or cytogenetic studies in mice with substance administration by the oral route (see Table 23). To conclude, this study by [redacted] (2007) was seriously flawed by severe toxicity that was completely unexpected and cannot be explained if the whole toxicological profile of glyphosate is taken into consideration. Either serious methodical mistakes have been made when the study was conducted or the test material was not glyphosate even though it was claimed as such. Both possibilities would turn the study completely unreliable and make it unsuitable for any regulatory use ″.

B.6.4.2.7. *In vivo studies in somatic cells – in vivo micronucleus, study 7*

Data point	CA 5.4.2/007
Report author	[redacted]
Report year	2006
Report title	Glyphosate Technical: Micronucleus Test In The Mouse
Report No	2060/014
Document No	Not reported
Guidelines followed in study	OECD 474 (1997); Commission Directive 2000/32/EC B.12 (2000), USA EPA, JMAFF
Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study 2000 polychromatic erythrocytes were evaluated (requirement of

	previous OECD guideline (1997)). Data on historical controls obtained in the testing laboratory were not provided. Acceptance criteria were not mentioned and evaluation criteria specified in the study report were inconsistent with those specified in the guideline. Individual animal data for clinical signs were not detailed in the study report. These deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: H05H016A, purity: 95.7 %) was tested for its genotoxic potential in mice using the micronucleus test. Prior to the micronucleus test, a preliminary toxicity study was performed. Due to severe clinical signs of toxicity and mortality at 800 and 1000 mg/kg bw, a maximum tolerated dose of 600 mg/kg bw was established and selected for the main study. No marked difference in toxicity was observed between male and female mice.

Based on these findings, the main micronucleus test was performed in male mice with dose levels of 150, 300 and 600 mg/kg bw. Seven mice per group received a single intraperitoneal injection at a constant dosage volume of 10 mL/kg bw. Similarly constituted groups received the vehicle (phosphate buffered saline, 7 mice/group) or the positive control (cyclophosphamide, 5 mice/group). Animals were sacrificed 24 (all treatment groups, vehicle and positive control animals) and 48 hours (vehicle control and high dose group) followed by preparation of bone marrow smears from the femoral bone marrow from each animal. After staining of the preparations, 2000 erythrocytes per animal were scored for the presence of micronucleated polychromatic and normochromatic cells. In addition, 1000 erythrocytes were counted to determine the percentage of polychromatic to normochromatic erythrocytes.

Intraperitoneal injection of the test item induced clinical signs of toxicity at ≥ 150 mg/kg bw in all animals of the 24 and 48 hour sacrifice. The observations comprised hunched posture, ptosis, ataxia and lethargy.

A statistically significant decrease in the ratio of polychromatic cells per 1000 erythrocytes was observed compared to the vehicle control in the 24-hour 600 mg/kg bw group, as well as a statistically non-significant decrease in the 48-hour 600 mg/kg bw group. This observation, as well as the occurrence of clinical signs demonstrated systemic absorption and confirmed exposure of the bone marrow.

There was a small but statistically significant increase in the incidence of micronucleated polychromatic erythrocytes in animals dosed with 600 mg/kg bw in the 24-hour sampling group when compared to the concurrent vehicle control group. However, the increase was very modest, within the range of the laboratory's historical control data and did not include any individual value that would not be acceptable for vehicle control animals. The observed response was attributed to a haematopoietic effect induced by the cytotoxic effect of the test material on the bone marrow rather than to any specific genotoxic effect and was therefore not considered to be of toxicological relevance. No statistically significant increase in the incidence of micronucleated polychromatic erythrocytes was observed after 48 hours in animals dosed at 600 mg/kg bw.

The positive control group showed a marked increase in the incidence of micronucleated PCE hence demonstrating capability of the test animals to show mutagenic effects in response to known mutagenic agents and confirming the sensitivity of the test.

Based on the results of the test and under the experimental conditions chosen, the test item was considered to be negative for clastogenicity/ aneugenicity in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

Identification: Glyphosate Technical

Description:	White crystalline solid
Lot/Batch #:	H05H016A
Purity:	95.7 %
Stability of test compound:	At room temperature stable until March 2008
Solvent (vehicle) used:	Phosphate buffered saline (PBS)

2. Control materials

Solvent (vehicle) control:	Phosphate buffered saline (PBS)
Positive control:	Cyclophosphamide, 50 mg/kg bw in PBS

3. Test animals:

Species:	Mouse
Strain:	CD-1
Sex:	Males
Source:	[redacted]
Age at study initiation:	Approx. 5 - 8 weeks
Weight at dosing:	21 - 29 g
Acclimation period:	At least 7 days
Diet/Food:	Certified Rat and Mouse Diet Code 5LF2, BCM (IPS Ldt., London UK), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	In groups up to seven in solid-floor polypropylene cages with wood flake bedding.

4. Environmental conditions:

Temperature:	19 - 25°C
Humidity:	30 - 70 %
Air changes:	Approximately 15/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	600, 800 and 1000 mg/kg bw
Concentrations:	60, 80 and 100 mg/mL
Dose volume:	10 mL/kg bw
Number of animals:	600 and 800 mg/kg bw: 1 male and 1 female 1000 mg/kg bw: 2 males and 2 females
Route of administration:	Intraperitoneal injection

b) Main micronucleus test

Dose levels:	150, 300 and 600 mg/kg bw
Concentrations:	15, 30 and 60 mg/mL
Dose volume:	10
Number of animals:	7 males/group
Route of administration:	Intraperitoneal injection

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 07 Jun – 20 Jul 2005
   Finalisation date: 13 Feb 2006

2. **Animal assignment and treatment:**

Preliminary toxicity study:
To examine the toxicity caused by glyphosate technical, single doses of 600, 800 and 1000 mg/kg bw were administered by intraperitoneal injection. One male and one female were used for the low and mid dose group whereas 2 males and females were used for the high dose group. Formulations were applied at a constant dosage volume of 10 mL/kg bw. The animals were observed for one hour following dosing and subsequently once daily for two consecutive days. Clinical signs of toxicity and mortality were recorded.

Main micronucleus test:
Groups of seven mice each received an intraperitoneal injection of 150, 300 and 600 mg/kg bw glyphosate technical, which was administered at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of control animals received the vehicle (PBS, 7 mice) or the positive control (50 mg/kg bw cyclophosphamide in PBS, 5 mice/group). All animals were observed for clinical signs of toxicity and mortality one hour post dosing and once daily thereafter and immediately prior to termination.

About 24 hours post-injection, the animals were sacrificed by cervical dislocation. For the high dose (600 mg/kg bw) as well as for the vehicle control group, a second group of 7 mice was sacrificed 48 hours post-injection.

3. **Slide preparation:**

Immediately following termination both femurs were dissected from each animal, aspirated with foetal calf serum and bone marrow smears prepared following centrifugation and re-suspension. The smears were air-dried, fixed in absolute methanol, stained in May-Grünwald/Giemsa, allowed to air-dry and coverslipped using mounting medium.

4. **Slide evaluation:**

Slides were randomly coded and examined under a light microscope at 1000x magnification. For the incidence of micronucleated cells, 2000 polychromatic erythrocytes (PCE) per animal were scored. In addition, the number of normochromatic erythrocytes (NCE) associated with 1000 erythrocytes was counted and also scored for incidences of micronuclei. After decoding the slide number, the percentage of PCE per 1000 erythrocytes was calculated.

5. **Statistics:**

The number of micronucleated PCE occurring in each test item treated group was compared to the number occurring in the corresponding solvent control. All data were statistically analysed following a $\sqrt{(x + 1)}$ transformation using Student's t-test (two tailed) and any significant results were confirmed using the one-way analysis of variance.

6. **Acceptance criteria:**

Acceptance criteria were not specified in the study report.

7. **Evaluation criteria:**

The test substance was judged positive for genotoxicity in vivo if the there was a dose-related, toxicologically relevant increase in the number of micronucleated PCE observed for either the 24 or 48 hour sacrifice time point when compared to solvent controls.
If these criteria were not fulfilled, then the test material was considered to be non-genotoxic under the conditions of the test.

A positive response for bone marrow toxicity was demonstrated when the dose group mean percentage of PCE per 1000 erythrocytes was shown to be statistically significantly lower than the concurrent vehicle control group.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

Severe clinical signs of toxicity were observed at 800 mg/kg bw and above. The animals showed hunched posture, lethargy, ataxia, ptosis, piloerection, tip toe gait, distended abdomen and hypothermia. At 600 mg/kg bw, clinical signs comprised hunched posture, ptosis, piloerection and ataxia of moderate severity. 2/2 and 2/4 animals at 800 and 1000 mg/kg bw, respectively, were killed in extremis due to the severity of clinical signs.

The test material showed no marked difference in its toxicity to male or female mice, it was therefore considered to be acceptable to use males only for the main test.

Based on the results of the preliminary toxicity study, 600 mg/kg bw was identified as maximum tolerated dose and chosen as highest dose level for the main micronucleus test.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

Clinical signs of toxicity were observed at 150 mg/kg bw and above in all animals. The observations comprised hunched posture, ptosis, ataxia and lethargy.

Evaluation of bone marrow slides:

A statistically significant decrease in the ratio of PCE (immature) to total erythrocytes compared to the vehicle control was observed in the 24-hour 600 mg/kg bw group, as well as a statistically non-significant decrease in the 48-hour 600 mg/kg bw group. The decreased ratio of PCE accompanied by the presence of clinical signs indicated systemic absorption and confirmed exposure of the bone marrow. Compared to solvent controls, a small but statistically significant increase in the incidence of micronucleated PCE was evident in the 24 hour 600 mg/kg bw group, but the values remained within the range of the laboratory's historical control data. The response was attributed to a haematopoetic effect due to bone marrow toxicity rather than to a specific genotoxic effect. The authors suggested that the increased erythropoiesis caused by test material toxicity might cause some cells to cycle more quickly than in the vehicle control animals and, therefore, there may also be less opportunity to repair spontaneously-occurring DNA damage before the final mitosis and enucleation, resulting in small increases in micronucleated cells. Therefore, the response was considered to have no toxicological significance. No statistically significant increase in the incidence of micronucleated PCE was observed in the remaining dose groups.

The positive control group showed a marked increase in the incidence of micronucleated PCE hence demonstrating validity and sensitivity of the conducted test to identify mutagenic effects in response to known mutagenic agents.

Table B.6.4.2.7-11: Mammalian Erythrocyte Micronucleus Test ([redacted], 2006), summary of genotoxicity data in mice

Treatment	Dose (mg/kg bw)	Sampling time	mPCE ± SD/2000 PCE	%PCE with micronuclei	%PCE/1000 Erythrocytes
PBS		24 h	1.3 ± 1.1	0.06 ± 0.06	38.46 ± 4.58
		48 h	2.0 ± 2.4	0.1 ± 0.12	36.01 ± 4.39
Test item	150	24 h	1.4 ± 0.8	0.07 ± 0.04	45.23 ± 6.12
	300	24 h	1.1 ± 1.1	0.06 ± 0.05	38.57 ± 8.69
	600	24 h	3.9 ± 1.5 *	0.19 ± 0.07*	27.71 ± 4.95**
	600	48 h	1.9 ± 2.1	0.09 ± 0.1	28.16 ± 14.23
CPA	50	24 h	60.6 ± 9.7 ***	3.03 ± 0.49 ***	51.46 ± 4.45

Table B.6.4.2.7-11: Mammalian Erythrocyte Micronucleus Test ([redacted], 2006), summary of genotoxicity data in mice

Treatment	Dose (mg/kg bw)	Sampling time	mPCE ± SD/2000 PCE	%PCE with micronuclei	%PCE/1000 Erythrocytes

mPCE: micronucleated polychromatic erythrocytes

SD: Standard deviation

PCE: polychromatic erythrocytes

* Statistical significance at $p < 0.05$ (*), at $p < 0.01$ (**) and at $p < 0.01$ (***) in the Student's t-test on transformed data

PBS: phosphate buffered saline, solvent control

CPA: cyclophosphamide, positive control

Table B.6.4.2.7-2: Historical control data for relative frequency categories of micronuclei per 1000 PCE*

24-h sampling			48-h sampling		
Frequency categories	**Groups**	**%**	**Frequency categories**	**Groups**	**%**
0.0 – 0.4	15	25	0.0 – 0.4	21	35
0.5 – 0.9	25	42	0.5 – 0.9	18	30
1.0 – 1.4	14	23	1.0 – 1.4	14	23
1.5 – 2.0	3	5	1.5 – 2.0	7	12
2.1 – 2.5	3	5	2.1 – 2.5	0	0

* Data from 60 studies

III. CONCLUSION:

Based on the results of the present study, glyphosate technical did not lead to clastogenic/aneugenic effects in bone marrow of male mice *in vivo*. Therefore, the test item was considered to be non-genotoxic under the conditions of the test.

Assessment and conclusion by applicant:

The study was performed under GLP conditions and in accordance with OECD guideline 474 (1997). Under the conditions of the test, the test material was considered negative for clastogenic/aneugenic effects in the bone marrow of male mice *in vivo*. The small but statistically significant increase in the incidence of micronucleated PCE in the 24 hour 600 mg/kg bw group remained within the range of the laboratory's historical control data. Moreover, no increase was seen after 48 hours. Thus, the response is interpreted as a haematopoietic effect due to bone marrow toxicity rather than to a specific genotoxic effect.

There were only minor deviations when compared to the currently valid OECD guideline 474 (2016), which were considered to be of minor degree. The number of polychromatic erythrocytes investigated per animal was 2000, which was the number recommended by the previous OECD guideline (1997). Further deviations were considered to not compromise the validity of the study. Therefore, the study was considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male mice i*n vivo* under the conditions of this test. Bone marrow toxicity was proven in this study in which glyphosate was administered by the intraperitoneal route.

The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations. It is noted that intraperitoneal administration is a disadvantage in the study since that cannot be an anticipated route for human exposure.

B.6.4.2.8. *In vivo studies in somatic cells – in vivo micronucleus, study 8*

Data point	CA 5.4.2/008
Report author	[redacted]
Report year	1999
Report title	A micronucleus study in mice for glifosate técnico [redacted]
Report No	[redacted]-G12.79/99
Document No	Not reported
Guidelines followed in study	Not specified. The test was conducted similary to OECD 474 (2016).
Deviations from current test guideline OECD 474 (2016)	Only 1000 polychromatic erythrocytes (PCE) per animal were evaluated for the presence of micronuclei, whereas at least 4000 PCE should be evaluated according to the current guideline OEC 474 (2016). Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity reported. Historical control data for the positive or vehicle controls were not provided. In addition, acceptance and evaluation criteria specified in the study report were inconsistent with those specified by the guideline. The deviations were not expected to significantly impact the study outcome.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: 037-919-113, purity: 95 %) was tested in a mouse bone marrow micronucleus assay. Prior to the micronucleus test, a preliminary toxicity study was performed. Based on the results of the toxicity study, the dose levels for the main mutagenicity study were selected to represent 25, 50 and 75 % of the LD_{50} in mice. The test item was dissolved in water and two intraperitoneal injections at dose levels of 187.5, 375 and 562.5 mg/kg bw/day were administered to two groups of 5 male and 5 female mice. The injections were performed on two consecutive days at an interval of 24 hours. A group of vehicle (water) and positive control animals (25 mg/kg bw/day cyclophosphamide in physiological solution) were treated in an identical manner.

All animals were sacrificed 24 hours after the second injection, followed by preparation and staining of bone marrow smears for all animals.

A total of 1000 polychromatic erythrocytes (PCE) and 1000 normochromatic erythrocytes (NCEs) were scored for the presence of micronuclei and the percentage of PCE to NCE was determined among 1000 erythrocytes.

Intraperitoneal injection of the test material did not induce clinical signs of toxicity in the mice. In addition, there was no increase in the number of micronucleated polychromatic or normochromatic erythrocytes when compared to the vehicle control and the ratio of PCE to NCEs was unaffected by treatment.

Treatment with the positive control cyclophosphamide revealed a marked increase in the incidence of micronucleated PCE, hence demonstrating capability of the test animals to show mutagenic effects in response to known mutagenic agents and confirming the sensitivity of the test.

Based on the results of the test and under the experimental conditions chosen, the test item was considered to be negative for induction of micronuclei in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: GLIFOSATE TÉCNICO [redacted]

Identification:	3578/99
Description:	White powder
Lot/Batch #:	037-919-113
Purity:	95 % (954.9 g/kg acid equivalent)
Stability of test compound:	The stability of the test item, storage conditions or expiry date were not reported. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	Water

2. Control materials:

Solvent (vehicle) control:	Water
Positive control:	Cyclophosphamide, 25 mg/kg bw/day in physiological solution

3. Test animals:

Species:	Mouse
Strain:	Swiss albino
Sex:	Male and female
Source:	[redacted]
Age at study initiation:	7 - 12 weeks
Mean weight at dosing:	30.22 g
Acclimation period:	At least 7 days
Diet/Food:	commercial pelleted diet (Labina, Purina), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	In groups of 5/sex on wood shavings in propylene rodent cages with stainless mesh lids

4. Environmental conditions:

Temperature:	20 – 24 °C
Humidity:	50 – 60 %
Air changes:	Not specified
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	250, 500, 1000 and 2000 mg/kg bw/day
Number of animals:	5/group
Route of administration:	Intraperitoneal injection

b) Main micronucleus test

Dose levels:	187.5, 375 and 562.5 mg/kg bw/day
Concentrations:	12.5, 25 and 37.5 mg/mL
Dose volume:	0.45 mL/30 g bw
Number of animals:	5/sex/group
Route of administration:	Intraperitoneal injection

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 28 Oct – 10 Dec 1999
   Finalisation date: 27 Dec 1999

2. **Animal assignment and treatment:**

Preliminary toxicity study:
The LD_{50} of the test material was determined in a preliminary toxicity study. Groups of five animals received two intraperitoneal injections of 250, 500, 1000 or 2000 mg/kg bw/day on two consecutive days. The animals were observed for four days and occurrence of mortality was recorded. Based on the findings of the study, the dose levels for the main micronucleus test were selected as 25, 50 and 75 % of the LD_{50}.

Main micronucleus test:
Groups of ten mice (5 per sex) received on two consecutive days (24 hours apart) two intraperitoneal injections of 187.5, 375 and 562.5 mg/kg bw/day glyphosate technical. Similar constituted groups of control animals were treated in an identical manner with the vehicle (water) or the positive control (25 mg/kg bw/day cyclophosphamide in physiological solution).

About 24 hours after the second injection, the animals were sacrificed by cervical dislocation.

3. **Slide preparation:**

Immediately after sacrifice both femurs were dissected from each animal, flushed with foetal calf serum and bone marrow smears prepared following centrifugation and re-suspension. The following day the smears were fixed in 70 % ethanol, air-dried and stained with Eosin methylene blue solution.

4. **Slide evaluation:**

Slides were randomly coded and examined under a light microscope at 1000x magnification. For each animal 1,000 polychromatic erythrocytes (PCE) and 1,000 normochromatic erythrocytes (NCE) were examined for the presence of micronuclei (MN). The relation PCE to NCEwere determined in the first 1,000 PCE or NCE enumerated.

5. **Statistics:**

Differences in the ratio of PCE to NCE and the incidence of micronucleated PCE and NCE per 1000 cells scored were compared using the Kruskal Wallis test for independent variables.

6. **Acceptance criteria:**

The test was considered valid only if the number of micronuclei in the vehicle control was within the range of the laboratory's historical control data.

7. **Evaluation criteria:**

The test substance was judged positive for genotoxicity in vivo if the following criteria were met:

- There was a reproducible and statistically significant positive response for at least one dose level and
- The increase in the number of micronuclei was at least twice the vehicle control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Information on the stability of the test substance in the vehicle was not given in the study report.

B. PRELIMINARY TOXICITY STUDY

Based on the occurrence of mortality within 4 days after treatment, the acute lethal dose for intraperitoneal toxicity (LD_{50}) was determined to be 750 mg/kg bw/day. Dose levels for the main micronucleus test were chosen to represent 25, 50 and 75 % of the LD_{50} obtained in the preliminary toxicity study.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:
Clinical signs of toxicity were not reported.

Evaluation of bone marrow slides:
No statistically or biologically significant increases in the incidence of micronucleated polychromatic erythrocytes or in the percentage of polychromatic erythrocytes were observed for the test substance either in male or female mice when compared to vehicle controls (water).
The positive control group revealed a statistically significant increase in the incidence of micronucleated PCE, demonstrating the sensitivity of the test.

Table B.6.4.2.8-1: A micronucleus study in mice ([redacted] 1999), Summary of genotoxicity data

Treatment	Dose (mg/kg bw/day)	Sampling time	mPCE ± SD /1000 PCE	PCE#	mNCE ± SD /1000 NCE	NCE#	PCE/NCE
Water		24 h	0.6	879	0	997.7	0.915
Test item	187.5	24 h	0.3	779.2	0.1	978.1	0.813
Test item	375.0	24 h	0.6	871.7	0.3	948.4	0.935
Test item	562.5	24 h	0.5	832.8	0.3	987.8	0.851
CPA	25.0	24 h	4.8*	648.9	2.0*	1029.5	0.63

mPCE: micronucleated polychromatic erythrocytes, mNCE: micronucleated normochromatic erythrocytes

* Statistical significance at $p < 0.05$ (*), at $p < 0.01$ (**) and at $p < 0.01$ (***) using the Kruskal Wallis test for independent variables

Mean number of PCE or NCE scored per 10 animals

CPA: cyclophosphamide, positive control

III. CONCLUSION

Based on the results of the present study, glyphosate technical did not increase the frequency of micronuclei in the bone marrow of mice in vivo. Thus, the test item was considered to be non-genotoxic under the conditions of the test.

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male and female mice *in vivo*.

The study was performed under GLP conditions and the experimental procedure was similar to the main criteria of OECD guideline 474 (2016), except for some deviations of minor degree. The number of polychromatic erythrocytes (PCE) evaluated was less than the 2000 PCE which are recommended by the current guideline (2016). However, there was no positive effect and no trend at all for induction of micronuclei, therefore it can be assumed that the outcome of the study would be the same if more cells were evaluated.

Dose levels were selected based on LD_{50} values which were determined in a preliminary toxicity study. Although clinical signs of systemic toxicity were not reported and there was no bone marrow toxicity evident, it can be assumed that due to the route of exposure (intraperitoneal injection) bone marrow exposure was achieved. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male and female mice i*n vivo* under the conditions of this test. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.2.9. *In vivo studies in somatic cells – in vivo micronucleus, study 9*

Data point	CA 5.4.2/009
Report author	[redacted]
Report year	1996
Report title	Glyphosate Acid: Mouse Bone Marrow Micronucleus Test
Report No	[redacted]/P/4954
Document No	Not reported
Guidelines followed in study	OECD 474 (1997); US EPA (1991) and EEC Annex V B.12 (1992)
Deviations from current test guideline OECD 474 (2016)	The currently defined highest dose for an administration of less than 14 days is exceeded (5000 mg/kg bw/day instead of 2000 mg/kg bw/day), however, no signs indicative of severe toxicity are reported. Only 2000 polychromatic erythrocytes were screened for the presence of micronuclei, instead of 4000, which are recommended by OECD 474 (2016). Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity observed, but dose levels exceeded limit concentrations specified in the current guideline. Historical control data established in the testing laboratory were not provided. In addition, acceptance criteria differed from those specified in the current guideline and evaluation criteria were not specified in the study report. The deviations were not expected to significantly impact the study outcome. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate acid (batch: P24, purity: 95.6 %) was tested for its genotoxic potential in mice using the micronucleus test. Prior to the micronucleus test, a preliminary toxicity study was conducted in order to identify the maximum tolerated dose in the animals.

Based on the results of the toxicity study, the test item was dissolved in physiological saline and groups of 5 male and 5 female mice received a single oral dose of 5000 mg/kg bw. Similarly constituted groups received the vehicle (physiological saline) or positive control (65 mg/kg bw cyclophosphamide in physiological saline) and were treated in an identical manner. The animals were sacrificed 24 or 48 hours after dosing, followed by preparation and staining of bone marrow smears.

A total of 2000 polychromatic erythrocytes (PCE) was evaluated for the presence of micronuclei. In addition, 1000 erythrocytes were counted to determine the percentage of PCE in the total erythrocyte population.

Oral administration of 5000 mg/kg bw did not induce any adverse reactions to treatment in any animal. In addition, there was no statistically significant change in the ratio of PCE among the total fraction of erythrocytes when compared to control animals, indicating that no relevant cytotoxicity occurred up to the maximum tolerated dose of 5000 mg/kg bw.

There was no statistically or biologically significant increases in the incidence of micronucleated PCE over the vehicle control values observed in either males or females at any sampling time investigated.

The positive control cyclophosphamide induced a statistically and biologically significant increase in the frequency of micronucleated polychromatic erythrocytes in both male and female mice, demonstrating the sensitivity of the test system and the capability of the test animals to respond to known mutagens.

Based on the results of the test and under the experimental conditions chosen, the test item was considered to be negative for induction of micronuclei in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate acid

Identification:	17984
Description:	White solid
Lot/Batch #:	P24
Purity:	95.6 %
Stability of test compound:	The stability of the test item under storage conditions (at ambient temperature in the dark) was guaranteed when used within the stated expiry date. The stability of the test item in solvent (vehicle) was not specified.
Solvent (vehicle) used:	Physiological saline

2. Control materials

Solvent (vehicle) control:	Physiological saline
Positive control:	Cyclophosphamide, 65 mg/kg bw in physiological saline

3. Test animals:

Species:	Mouse
Strain:	CD-1
Sex:	Male and female
Source:	[redacted]
Age at study initiation:	6-7 weeks
Weight at dosing:	22.8 – 37.6 g
Acclimation period:	At least 5 days
Diet/Food:	CT1 (supplied by Special Diets Services, Stepfield, Witham, Essex, UK, *ad libitum*
Water:	Water, supplied by an automated watering system, *ad libitum*
Housing:	In groups of 5/sex per cage on mobile mouse cage racks

4. Environmental conditions:

Temperature:	19-23 °C
Humidity:	40-70 %
Air changes:	15/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	5000 mg/kg bw
Number of animals:	5 /sex / group
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	5000 mg/kg bw
Dose volume:	20 mL/kg bw
Number of animals:	5 /sex / group
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 11 Dec 1995 – 30 Jan 1996
Finalisation date: 21 Mar 1996

2. Animal assignment and treatment:

Preliminary toxicity study:
The maximum tolerated dose (MTD), was determined in a preliminary toxicity study (termed as "Phase 1" in the study report). 5 male and 5 female mice received a single dose of 5000 mg/kg bw by oral gavage. Based on the occurrence of mortality and clinical signs of toxicity within 4 days after treatment, the maximum tolerated dose was determined.

Main micronucleus test:
Groups of ten mice (5 per sex) were given a single oral dose of 5000 mg/kg bw by oral gavage, representing the maximum tolerated dose. Similar constituted groups of control animals were treated in an identical manner with the vehicle (physiological saline) or the positive control (65 mg/kg bw cyclophosphamide in physiological saline).

The animals were observed for adverse reactions to treatment and sacrificed 24 and 48 hours after dosing by asphyxiation of halothane followed by cervical dislocation.

3. Slide preparation:

Immediately after sacrifice both femurs were dissected from each animal. The iliac end of the femur was removed and a fine paint brush wetted in a solution of 6 % (w/v) albumin in saline was dipped into the marrow canal. From each marrow, four smears in total were painted on appropriately labelled, clean and dry microscope slides. The slides were allowed to air-dry and stained with polychrome methylene blue and eosin.

4. Slide evaluation:

Slides were coded and scored by microscopy. For each animal 2000 polychromatic erythrocytes (PCE) were examined for the presence of micronuclei. In addition, 1000 erythrocytes were counted to determine the percentage of PCE in the total erythrocyte population.

5. Statistics:

The incidence of micronucleated PCE and the percentage of PCE in the erythrocyte sample were considered by analysis of variance at 24 and 48 hours, separately for males and females. Prior to analysis, the data for the incidence of micronucleated PEC was transformed using a square root transformation and the data for the ratio of PCE among the total number of erythrocytes was transformed using the double arcsin transformation of Freeman and Tukey (1950)[10]. Each treatment group mean was compared with the control group mean at the corresponding sample time using a one-sided Student's t-test based on the error mean square in the analysis.

6. Acceptance criteria:

The test was considered valid if the following criteria were met:

[10] Freeman M.F. and Tukey *J*. W. (1950). Transformations related to the angular and the square root. Annals of Maths Stats 21, 607

- The positive control substance induced a significant elevation in micronucleated polychromatic erythrocytes when compared to vehicle controls.
- The test material was tested at a level that caused a decrease in the percentage of erythrocytes (indicating a cytotoxic effect on the bone marrow) or at the maximum tolerated dose level.

7. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

During the four days observation period, no patterns of lethality or severe toxicity were observed. As no clinical signs and no lethality was evident at the limit dose level, 5000 mg/kg bw were taken to represent the maximum tolerated dose for males and females.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

No adverse reactions to treatment were observed for either males or females.

Evaluation of bone marrow slides:

No statistically significant differences in the percentage of polychromatic erythrocytes was observed for glyphosate acid treated animals when compared to control animals, indicating that no relevant cytotoxicity occurred up to the maximum tolerated dose of 5000 mg/kg bw.

In addition, no statistically or biologically significant increases in the incidence of micronucleated polychromatic erythrocytes over the vehicle control values were observed in either males or females at either sampling time investigated.

The positive control cyclophosphamide induced a statistically and biologically significant increase in the frequency of micronucleated polychromatic erythrocytes in both male and female mice, demonstrating the sensitivity of the test system.

Table B.6.4.2.9-1: Mouse Bone Marrow Micronucleus Test ([redacted], 1996), genotoxicity results

Treatment	Dose (mg/kg bw)	Sampling time	Males		Females	
			mPCE ± SD /1000 PCE	mean% PCE ± SD	mPCE ± SD /1000 PCE	mean% PCE ± SD
Saline	20 mL/kg	24 h	1.6 ± 0.8	46.0 ± 4.1	1.4 ± 0.7	46.6 ± 1.9
Saline	20 mL/kg	48 h	1.7 ± 1.3	49.8 ± 4.8	0.7 ± 0.6	46.4 ± 4.4
Test item	5000	24 h	2.1 ± 1.6	46.4 ± 4.3	2.1 ± 2.5	42.3 ± 6.2
Test item	5000	48 h	2.1 ± 1.9	48.4 ± 4.0	0.8 ± 0.8	45.8 ± 2.9
CPA	65	24 h	22.2 ± 6.1 **	46.5 ± 2.0	23.3 ± 4.9 **	46.0 ± 4.1

mPCE: micronucleated polychromatic erythrocytes

* Statistical significance at $p < 0.05$ (*), at $p < 0.01$ (**) and at $p < 0.01$ (***) in the Student's t-test (one sided) in transformed data

CPA: cyclophosphamide, positive control

III. CONCLUSION:

Based on the results of the present study, glyphosate acid has no clastogenic/aneugenic activity in bone marrow of male and female mice when tested up to the limit dose of 5000 mg/kg bw. Thus, the test item was considered to be non-genotoxic under the conditions of the test.

Assessment and conclusion by applicant:
Negative for clastogenic/aneugenic effects in the bone marrow of male and female mice *in vivo*.

The study was conducted under GLP conditions and in line with OECD guideline 474 (1997). There were a number of deviations when compared to the currently valid OECD guideline 474 (2016), all of them considered to be of minor degree. Only 2000 polychromatic erythrocytes were investigated, which was the number required according to the previous guideline (1997) that was in force when the test was conducted. Bone marrow toxicity, indicated by a shifted polychromatic to normochromatic erythrocyte ratio or systemic toxicity were not observed. However, test item doses included the dose level of 5000 mg/kg bw, which exceeds the limit value specified in the current guideline (2016). The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male and female mice *in vivo*. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.2.10. *In vivo studies in somatic cells – in vivo micronucleus, study 10*

Data point	CA 5.4.2/010
Report author	[redacted]
Report year	1993
Report title	Glyphosate technical: Mutagenicity - Micronucleus Test in Swiss Albino Mice
Report No	889-MUT.MN
Document No	Not reported
Guidelines followed in study	OECD 474 (1983)
Deviations from current test guideline OECD 474 (2016)	The currently defined highest dose for an administration of less than 14 days is exceeded (5000 mg/kg bw/day instead of 2000 mg/kg bw/day), however, no signs indicative of severe toxicity are reported. According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study 2000 erythrocytes (RBC) were evaluated, including approx. 800 – 1100 polychromatic erythrocytes per animal. In addition, no data on proficiency and/or historical control data were provided. Acceptance and evaluation criteria were not specified in the study report. The deviations were not expected to significantly impact the study outcome. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed. ***AGG notes that a discrepancy was seen regarding the batch number and purity reported in the study report and in the certificate of analysis that is attached to the study report. The applicant is asked to clarify this.***
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a

	Conclusion AGG: The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations and the t-test used.

Glyphosate technical (batch: FSG 03090, purity: 96.8%) was assessed for its ability to induce cytogenetic effects in Swiss albino mice. Based on the results of a preliminary range-finding study, dose levels for the micronucleus test were selected. Groups of 5 mice/sex were treated by oral gavage at dose levels of 50, 500 and 5000 mg/kg bw/day at a constant dosage volume of 10 mL/kg bw for two consecutive days. Similar constituted groups received the vehicle (refined groundnut oil, 10 mice/sex) or the positive control cyclophosphamide (100 mg/kg bw/day, 5 mice/sex).

The animals were observed for clinical signs of toxicity and mortality twice daily and the time of onset, duration and severity were recorded. In addition, the body weight of the individual animals was recorded on study Days 1, 2 and at sacrifice. 24 hours after the second dose administration the animals were sacrificed and femoral bone marrow smears were prepared for each animal. At least 2000 erythrocytes per animal were scored for the presence of micronuclei and the ratio of polychromatic to normochromatic erythrocytes was determined.

Oral administration of glyphosate technical did not induce any clinical signs of toxicity or mortality. However, at sacrifice, animals of the 500 and 5000 mg/kg bw/day group had lost body weight. The ratio of polychromatic to normochromatic erythrocytes (NCEs) of glyphosate treated mice was unaffected and comparable to those of mice treated with the vehicle, indicating that no bone marrow toxicity had occurred.

In female mice, there was a slight but statistically significant increase in the frequency of micronucleated polychromatic erythrocytes (PCEs) at the highest dose level of 5000 mg/kg bw/day when compared to control animals. There was no increase in the frequency of micronucleated PCEs at the low and the mid dose level and no effect in male mice at any dose level.

The frequency of micronucleated PCE, NCE and total erythrocytes in the solvent control animals were comparably low for all animals. The positive control cyclophosphamide showed the expected statistically significant increase in the incidence of micronucleated PCE and NCE in both sexes and a high degree of bone marrow cytotoxicity evident as markedly affected PCE/NCE ratio, demonstrating the sensitivity of the test system.

Under the conditions of the test and based on the experimental findings, glyphosate technical was considered weakly positive for induction of micronuclei in female mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate (N-(Phosphonomethyl) glycine)

Identification:	C140500
Description:	Odorless white crystals
Code:	FSG 03090 H/05, March 1990
Purity:	96.8 %
Stability of test compound:	The stability of the test item at storage conditions (at ambient temperature) was guaranteed until the expiry date Jul 1992. The stability of the test item in solvent was not specified. Dosing formulations were prepared fresh prior to treatment. Homogeneity of the test compound suspension was maintained by constant stirring/mixing in mortar during treatment.
Solvent (vehicle) used:	Refined groundnut (peanut) oil

2. Control materials

Solvent (vehicle) control:	Refined groundnut (peanut) oil
Positive control:	Cyclophosphamide, 100 mg/kg bw/day

3. Test animals:

Species:	Mouse
Strain:	Swiss albino
Sex:	Male and female
Source:	[redacted]
Age at study initiation:	8 - 10 weeks
Weight at dosing:	25 - 35 g
Acclimation period:	At least one week
Diet/Food:	Gold Mohur pelleted mice feed (Lipton India Ltd., Bangalore, India), *ad libitum*
Water:	Protected, deep borewell water, passed through activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter-cum-purifier, *ad libitum*
Housing:	In groups of 5/sex in sterilised standard polypropylene cages measuring 290 x 220 x 140 mm with paddy husk bedding

4. Environmental conditions:

Temperature:	22 ± 3 °C
Humidity:	40 - 70 %
Air changes:	Approximately 12-15/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

Dose levels:	50, 500 and 5000 mg/kg bw/day
Concentrations:	5, 50, 500 mg/mL
Dose volume:	10 mL/kg bw
Number of animals:	5/sex/group (test item and positive control) and 10/sex/group (vehicle control)
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 10 Aug – 10 Dec 1991
Finalisation date: 06 May 1993

2. Animal assignment and treatment:

Groups of five mice per sex received two doses of 50, 500 and 5000 mg/kg bw/day on two consecutive days. The test item was dissolved in refined groundnut (peanut) oil and administered via oral gavage at a constant dosage volume of 10 mL/kg bw. Groups of 10 mice/sex received the vehicle. Groups of 5 mice/sex were treated with the positive control (100 mg/kg bw/day cyclophosphamide).

The animals were observed for clinical signs of toxicity and mortality twice daily and the time of onset, duration and severity were recorded. In addition, the body weight of the individual animals was recorded on study Days 1, 2 and at sacrifice.

24 hours after the last treatment, the animals were sacrificed by cervical dislocation and bone marrow smears were prepared.

3. Slide preparation:

After sacrifice, the femur bones from both sides were removed, dissected and marrow cells were flushed out with sterile physiological saline. The cells were centrifuged, re-suspended in physiological saline, smeared evenly on

microscopic glass slides and air-dried. 4-6 slides were prepared for each animal. Finally, the slides were fixed in methanol for 30 minutes by immersing them in a coplin jar and stained with May-Grünwald/Giemsa. Afterwards, the slides were blow-dried, immersed in xylene and coverslip mounted with DPX.

4. Slide evaluation:

For each animal a minimum of 2000 erythrocytes was scored for the incidence of polychromatic (PCE) and normochromatic (NCE) erythrocytes and for the presence of micronuclei. The following data were recorded:

- Total red blood cells /erythrocytes scored
- Number of PCEs differentiated
- Number and percentage of PCE with micronuclei
- Number of NCE differentiated
- Number and percentage of NCE with micronuclei
- Number and percentage of total red blood cells with micronuclei
- Ratio of PCE/NCE

5. Statistics:

The data was analysed group-wise and sex-wise using one way Anova and t-test for unequal number of observations for the percentage of micronucleated cells in the treated and control groups at $p \leq 0.05$ (indicated by a $ in the summary below). The t-test was further used for dose-effect relationship (when required at $p \leq 0.05$ and 0.10; indicated by a * in the summary below).

6. Acceptance criteria:

Acceptance criteria were not specified in the study report.

7. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in this study, as not compulsory by the test guideline.

B. MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

There were no compound- and dose-related signs of systemic toxicity observed.

Body weight:

At sacrifice, marginal body weight loss was noted in the animals of the mid (500 mg/kg bw/day, -1.9 % in males and -2.0 % in females) and high dose group (5000 mg/kg bw/day, -4.3 % in males and -1.0 % in females), respectively.

Evaluation of bone marrow slides:

There was no difference in the ratio of polychromatic (PCE) to normochromatic erythrocytes (NCE) for any of the glyphosate treated groups when compared to solvent control animals, indicating that no bone marrow toxicity had occurred.

In female mice, there was a slight but statistically significant increase in the frequency of micronucleated polychromatic erythrocytes (PCEs) at the highest dose level of 5000 mg/kg bw/day when compared to control animals. A dose effect relationship was evident in those animals.

There was no increase in the frequency of micronucleated PCEs at the low, mid and high dose level in males or as combined sex data.

The toxicological significance of the glyphosate-related increase of micronucleated PCEs in one sex was considered doubtful, as the variation in the percentage of micronucleated PCEs was considerably high among female dose groups as compared to the control.

The frequency of micronucleated PCE, NCE and total erythrocytes in the solvent control animals were comparably low for all animals. The positive control cyclophosphamide showed the expected statistically significant increase in the incidence of micronucleated PCE and NCE in both sexes and a high degree of bone marrow cytotoxicity evident as markedly affected PCE/NCE ratio, demonstrating the sensitivity of the test system.

Table B.6.4.2.10-1: Mutagenicity – Micronucleus test in Swiss Albino Mice [redacted], 1993), summary of genotoxicity data, male mice

Treatment	Dose (mg/kg bw/day)	Number of animals	Sampling time	Males				
				mPCE ± SD /2000 RBC [X]	mean% PCE ± SD [X]	mNCE ± SD /2000 RBC [X]	mean% NCE ± SD [X]	PCE/NCE ratio
Vehicle		10	24 h	6.40 ± 5.56	0.69	6.60 ± 5.70	0.62	1:1.1
Test item	50	5	24 h	7.60 ± 4.72	0.84	6.60 ± 3.29	0.64	1:1.1
	500	5	24 h	7.20 ± 3.83	0.73	2.80 ± 2.77	0.22	1:1.2
	5000	5	24 h	9.60 ± 2.41	0.89	6.40 ± 1.82	0.47	1:1.3
CPA	100	5	24 h	21.10 ± 6.42	2.33[$]	20.20 ± 5.89	1.18[$]	1:1.9

mPCE: micronucleated polychromatic erythrocytes; mNCE: micronucleated normochromatic erythrocytes
RBC: red blood cells, 2000 RBCs were scored for mPCE and mNCE; based on the values reported in the study report approx. 800 – 1100 PCE /animal were scored for the presence of micronuclei

[$]: significantly higher than control by contingency test;

[X]: mPCE and mNCE values calculated based on number of PCE and NCE, raw data provided in study report

Table B.6.4.2.10-2: Mutagenicity – Micronucleus test in Swiss Albino Mice ([redacted], 1993), summary of genotoxicity data, female mice

Treatment	Dose (mg/kg bw/day)	Number of animals	Sampling time	Females				
				mPCE ± SD /2000 RBC [X]	mean % PCE ± SD [X]	mNCE ± SD /2000 RBC [X]	mean% NCE ± SD [X]	PCE/NCE ratio
Vehicle		10	24 h	5.10 ± 3.35	0.51	4.70 ± 2.11	0.39	1:1.2
Test item	50	5	24 h	2.60 ± 1.67	0.28	1.80 ± 0.84	0.15	1:1.3
	500	5	24 h	5.40 ± 4.51	0.52	2.80 ± 1.48	0.23	1:1.2
	5000	5	24 h	10.60 ± 5.08	1.05[$*]	6.00 ± 2.00	0.46	1:1.3
CPA	100	5	24 h	17.60 ± 5.81	2.39[$]	23.40 ± 2.70	1.65[$]	1:1.9

mPCE: micronucleated polychromatic erythrocytes; mNCE: micronucleated normochromatic erythrocytes
RBC: red blood cells, 2000 RBCs were scored for mPCE and mNCE; based on the values reported in the study report approx. 800 – 1100 PCE/ animal were scored for the presence of micronuclei

[$]: significantly higher than control by contingency test; *: t-test for dose response relationship significant at $p < 0.1$

[X]: mPCE and mNCE values calculated based on number of PCE and NCE, raw data provided in study report

Table B.6.4.2.10-3: Mutagenicity – Micronucleus test in Swiss Albino Mice ([redacted], 1993), summary of genotoxicity data, both sexes combined

Treatment	Dose (mg/kg bw/day)	Number of animals	Sampling time	Combined sex				
				mPCE ± SD /2000 RBC [X]	mean% PCE ± SD [X]	mNCE ± SD /2000 RBC [X]	mean% NCE ± SD [X]	PCE/NCE ratio
Vehicle		20	24 h	5.75 ± 4.52	0.6	5.65 ± 4.30	0.5	1:1.2
Test item	50	10	24 h	5.10 ± 4.25	0.55	4.20 ± 3.39	0.38	1:1.2
	500	10	24 h	6.30 ± 4.06	0.62	2.80 ± 2.10	0.23	1:1.2
	5000	10	24 h	10.10 ± 3.78	0.96[*]	6.20 ± 1.81	0.47	1:1.3
CPA	100	10	24 h	19.40 ± 6.08	2.36[$]	21.80 ± 4.64	1.39[$]	1:1.9

mPCE: micronucleated polychromatic erythrocytes; mNCE: micronucleated normochromatic erythrocytes
RBC: red blood cells, 2000 RBCs were scored for mPCE and mNCE; based on the values reported in the study report approx. 800 – 1100 PCE/ animal were scored for the presence of micronuclei

[$]: significantly higher than control by contingency test; *: t-test for dose response relationship significant at $p < 0.1$

[X]: mPCE and mNCE values calculated based on number of PCE and NCE, raw data provided in study report

III. CONCLUSION:

Based on the experimental findings glyphosate technical induced micronuclei in female mice at a dose level of 5000 mg/kg bw/day. No increase in micronuclei formation was observed at lower dose levels in females or in males at any dose level. In conclusion, the test substance was considered weakly positive for clastogenic/aneugenic effects in female mice and negative in male mice *in vivo*.

Assessment and conclusion by applicant:

Based on the experimental findings the test item was considered weakly positive for clastogenic/aneugenic effects in the bone marrow of female mice *in vivo*. The study was performed according to OECD guideline 474 (1983) and under GLP conditions. The number of evaluated polychromatic erythrocytes was far below the recommendation of the current OECD guideline (2016), which resulted in rather high standard deviations. Due to missing historical control data, the biological significance of the weak positive result observed in females is unclear. Besides this, only minor deviations to the currently valid OECD guideline are present. Therefore, the study is considered to provide supporting information. Further deviations were considered to not compromise the outcome of the study.

Assessment and conclusion by RMS: The RMS is on the opinion that the test substance can be considered negative for clastogenic/aneugenic effects in male and equivocal in female mice *in vivo*. The RMS does not agree with the applicant's conclusion that the test substance was considered weakly positive for clastogenic/aneugenic effects in female mice as the standard deviations are very high. Furthermore, the additional t-test for dose response relationship significant at $p < 0.1$ is not suitable, either $P < 0.05$ or < 0.01 should be used. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.
It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.

B.6.4.2.11. *In vivo studies in somatic cells – in vivo micronucleus, study 11*

Data point:	CA 5.4.2/011
Report author	[redacted]

Report year	1990
Report title	Agrichem glyphosate active: OECD 474 micronucleus test in the mouse
Report No	300/3
Document No	Not reported
Guidelines followed in study	OECD TG 474 (1981), EEC Commission Directive 84/449 Method B12 (1984)
GLP	Yes
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate technical (batch: 0190A, purity: not reported) was tested for its genotoxic potential in BKW mice in a micronucleus test. Based on the results of a preliminary toxicity study, in which no mortality was observed up to an oral dose level of 5000 mg/kg bw, a dose of 4000 mg/kg bw was selected for the main mutagenicity test. The test item was dissolved in water and administered by oral gavage to three groups of mice for 3 sampling time points (24, 48 and 72 h after administration). Similar constituted groups received the solvent (distilled water) or the positive control (50 mg/kg bw cyclophosphamide). The animals were observed for clinical signs of toxicity and death one hour after dosing and once daily thereafter. Sampling of the vehicle control occurred at 24, 48 and 72 hours after treatment. For the positive control, bone marrow was sampled 24 hours following treatment. Smears were prepared from the femoral bone marrow of each animal and 1000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei. In addition, the number of micronucleated normochromatic erythrocytes (NCE) associated with 1000 PCE were counted and scored for the incidence of micronuclei.
Short description of results:	Upon treatment with glyphosate technical, 4/10, 2/10 and 1/10 animals died prematurely of the 24 hour, 48 hour and 72 hour sampling group, respectively. Substitute animals were included for the respective dose groups. Except one substitute animal of the 48 hour sampling group, all animals survived until scheduled sacrifice. Thus, the toxicological relevance of the observed mortality is unclear taking into consideration the survival rate of the substitute animals and the test animals in the preliminary study. Clinical signs of toxicity were observed in one animal and comprised hunched posture, lethargy, piloerection, decreased respiratory rate, ptosis and ataxia. The majority of substitute animals showed minor clinical signs of toxicity. When compared to solvent controls, the ratio of PCE/NCE was not affected by treatment, indicating that glyphosate did not induce bone marrow toxicity. There was no statistically significant increase in the incidence of micronucleated PCE or NCE for any sampling time point when compared to their concurrent vehicle controls. However, the respective standard deviations indicate a rather high heterogeneity in results. The positive control cyclophosphamide showed a marked increase in the incidence of micronucleated PCE and a small elevation of the PCE/NCE ratio, hence confirming the sensitivity of the test. Under the conditions of the test, the results indicate that glyphosate technical is negative for cytogenetic effects in bone marrow in mice *in vivo*.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG**: The study was considered not acceptable due to a large number of guideline deviations. According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study only 1000 polychromatic erythrocytes were evaluated. No data on proficiency and/or historical control data were provided and the purity of the test material was not reported. The highest tested dose level of 4000 mg/kg bw exceeds the currently defined maximum dose of 2000 mg/kg bw for administration periods < 14 days. Strong mortality was noted in

	the main mutagenicity study among various test groups, although a dose-response relationship was not evident and the deaths were not clearly attributable to treatment. The mortality observed was inconsistent with observations in other studies. In addition, evaluation criteria were inconsistent with those specified in the current guideline (2016). Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as described by GRG.

B.6.4.2.12. *In vivo studies in somatic cells – in vivo micronucleus, study 12*

Data point	CA 5.4.2/012
Report author	[redacted]
Report year	1991
Report title	Mutagenicity test: Micronucleus test with Glyphosate, batch 206-JaK-25-1
Report No	12324
Document No	Not reported
Guidelines followed in study	OECD 474 (1983), US EPA FIFRA 84-2
Deviations from current test guideline OECD 474 (2016)	Body weights and clinical signs have not been described in the study report. The currently defined highest dose for an administration of less than 14 days is exceeded (5000 mg/kg bw/day instead of 2000 mg/kg bw/day), however, no signs indicative of severe toxicity are reported. According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study 2000 polychromatic erythrocytes were evaluated (requirement of previous OECD guideline (1983)). In addition, the percentage of polychromatic erythrocytes among total erythrocytes was determined for 200 erythrocytes instead of 500 erythrocytes. Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio or systemic toxicity were not observed. In addition, no 24-hour control animals were included (first sampling time point). No data on proficiency and/or historical control data were provided. Acceptance and evaluation criteria were not specified in the study report. The deviations were not expected to significantly impact the study outcome. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** The study is considered to be acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: 206-JaK-25-1, purity: 98.6 %) was tested for its clastogenic/aneugenic potential in NMRI mice in a micronucleus test. Based on the results of an initial toxicity study in which no signs of toxicity became evident, the main micronucleus test was performed at a single dose level of 5000 mg/kg bw.

Three groups of 5 animals/sex received a single dose of the test item, dissolved in 0.5 % carboxymethyl cellulose and administered at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of 5 mice/sex received the positive (cyclophosphamide, 30 mg/kg bw) or vehicle control (0.5 % carboxymethyl cellulose in distilled water).

Bone marrow sampling of the test item treated groups was performed 24, 48 and 72 hours after treatment. Positive and vehicle control animals were sacrificed 24 and 48 hours after dosing, respectively. Smears were prepared from the femoral bone marrow of each animal and 2000 immature erythrocytes (PCE) per animal were scored for the

presence of micronuclei. The percentage of PCE in 200 erythrocytes was calculated. In addition, the number of micronucleated normochromatic erythrocytes (NCEs) was determined.

The ratio of PCE to NCEs indicates that no bone marrow toxicity was evident in any of the tested conditions.

The incidence of micronucleated polychromatic erythrocytes after treatment with glyphosate was comparable between control and treatment groups for any sampling time point. The frequency of micronuclei in the solvent and positive control groups were in accordance with the laboratory's historical control data, confirming validity and sensitivity of the test. Based on the experimental findings, glyphosate technical is negative for cytogenetic effects in bone marrow in mice *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

Identification:	Glyphosate Technical
Description:	White powder
Lot/Batch #:	206-JaK-25-1
Purity:	98.6 %
Stability of test compound:	The stability of the test item, storage conditions or expiry date were not reported. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	0.5 % carboxymethyl cellulose in distilled water

2. Control materials

Solvent (vehicle) control:	0.5 % carboxymethyl cellulose in distilled water
Positive control:	Cyclophosphamide, 30 mg/kg bw

3. Test animals:

Species:	Mouse
Strain:	Bom:NMRI (NMRI SPF mice)
Sex:	Male and female
Source:	[redacted]
Age at study initiation:	7 - 10 weeks
Weight at dosing:	26 - 31 g
Acclimation period:	5 days
Diet/Food:	Altromin 1314 (Chr. Petersen Ltd., Ringsted, Denmark), *ad libitum*
Water:	Tap water acidified with hydrochloric acid to pH 2.5, *ad libitum*
Housing:	In groups of 5/sex in Macrolon type III cages measuring 420 x 260 x 150 mm with softwood sawdust bedding.

4. Environmental conditions:

Temperature:	21 ± 3 °C
Humidity:	55 ± 15 %
Air changes:	Approximately 10/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	5000 mg/kg bw
Concentrations:	Not specified
Dose volume:	10 mL/kg bw
Number of animals:	5/sex/group
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	5000 mg/kg bw
Concentrations:	500 mg/mL
Dose volume:	10 mL/kg bw
Number of animals:	5/sex/group
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 24 Jan – 01 Feb 1991
Finalisation date: 12 Sep 1991

2. Animal assignment and treatment:

Preliminary toxicity study:
Glyphosate was expected to be of low toxicity. To determine the maximum tolerated dose, a single dose of 5000 mg/kg bw was administered by oral gavage to 4 male and 5 female mice. A group of 3 mice further received the vehicle and served as control. The test substance or the vehicle was applied at a constant dosage volume of 10 mL/kg bw. Bone marrow samples were prepared 24, 48 and 72 hours after dosing from 3 mice per sampling time point and 48 hours after dosing for the control group. To assess bone marrow toxicity upon treatment with the test item, the percentage of polychromatic erythrocytes among the total of erythrocytes was determined for 200 erythrocytes for each animal. The amount of PCE was comparable between the control and test group.

Based on these results, 5000 mg/kg bw was selected as dose level for the main micronucleus test.

Main micronucleus test:
Groups of five mice per sex were administered a single dose of 5000 mg/kg bw by oral gavage at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of 5 mice/sex received the vehicle (0.5 % carboxymethyl cellulose) or the positive control (30 mg/kg bw cyclophosphamide).
Animals of the test group were sacrificed by cervical dislocation at 24, 48 and 72 hours after treatment and bone marrow smears were prepared. Positive control animals were sacrificed 24 hours after treatment, the vehicle control group was sacrificed 48 hours after dosing.

3. Slide preparation:

Immediately after sacrifice, the right femoral bone was dissected, the proximal end of the femur was cut and marrow cells were flushed out with foetal calf serum. After whirl-mixing, the bone marrow suspensions were centrifuged and smears were prepared. The specimens were fixed in methanol and stained with May-Grünwald/Giemsa

4. Slide evaluation:

Slides were randomly coded and examined by microscopical analysis. About 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei. The percentage of PCE in 200 erythrocytes was calculated. In addition, the number of micronucleated normochromatic erythrocytes (NCE) was determined during the counting of 2000 PCE.

5. Statistics:

The number of micronucleated PCE in the test group were compared to the number found in the vehicle group. Statistical analysis was performed using the one-way analysis of variance performed on the values transformed to normal scores according to Blom's method[11].

[11] Blom (1958): Statistical Estimates and Transformed Beta Variables, New York: John Wiley and Sons, Inc

6. Acceptance criteria:

Acceptance criteria were not specified in the study report.

7. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

Clinical signs of systemic toxicity have not been described in the study report. Bone marrow toxicity, evident as a shifted ratio of polychromatic erythrocytes has not been observed. The percentage of PCE was between 34 and 43 % for both, test and control group. Based on the results of the preliminary toxicity study, 5000 mg/kg bw was selected as dose level for the main micronucleus test.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality reported.

Clinical signs of toxicity:

Clinical signs of toxicity were not described in the study report.

Evaluation of bone marrow slides:

There was no difference in the ratio of polychromatic to normochromatic erythrocytes when compared to vehicle controls for any sampling time point, demonstrating that there was no bone marrow toxicity evident. Values were in the range of 37.7 – 42.2 % and fell into the normal range of the laboratory (data not shown).

There was no statistically significant increase in the incidence of micronucleated PCE in test item-treated animals at any sampling time point when compared to vehicle control. The frequency of micronuclei in the solvent and positive control groups were in accordance with the laboratory's historical control data, confirming the sensitivity of the test and demonstrating the capability of the test animals to respond to mutagenic substances.

Table B.6.4.2.12-1: Mutagenicity test: Micronucleus test with Glyphosate ([redacted], 1991b), summary of genotoxicity data

Treatment	Dose (mg/kg bw)	Sampling time	Males		Females	
			mPCE ± SD /2000 PCE	mean% PCE ± SD /200 erythrocytes	mPCE ± SD /2000 PCE	mean% PCE ± SD /200 erythrocytes
CMC	0.5 %	48 h	3.00 ± 1.41	36.00 ± 5.15[#]	2.40 ± 0.55	39.80 ± 1.30[#]
Test item	5000	24 h	3.40 ± 1.14	35.60 ± 2.97	3.00 ± 1.41	40.00 ± 3.32[#]
	5000	48 h	2.20 ± 0.84	37.00 ± 2.24	3.40 ± 1.67	40.40 ± 1.95
	5000	72 h	1.80 ± 1.30	37.40 ± 3.44	1.60 ± 1.14	37.80 ± 3.63
CPA	30	24 h	48.60 ± 4.98	41.20 ± 3.03	47.80 ± 9.96	43.00 ± 5.70

Mean values and standard deviations (SD) were calculated based on the raw data given in the study report.

mPCE: number of micronuclei observed in 2000 PCE

%PCE: PCE in percent of total erythrocytes

[#] Calculations were performed with raw data values of poor legibility.

CMC: carboxymethyl cellulose, solvent control; CPA: cyclophosphamide, positive control

III. CONCLUSION

Based on the experimental findings glyphosate technical is negative for cytogenetic effects in bone marrow in mice *in vivo*.

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of male and female mice i*n vivo*.

The study was performed under GLP conditions and in accordance with OECD guideline 474 (1983). When compared to the currently valid OECD guideline 474 (2016), a number of deviations became evident. The number of polychromatic erythrocytes investigated was 2000/animal, corresponding to the number required by the previous guideline (1997). In addition, the percentage of polychromatic erythrocytes among total erythrocytes was determined for 200 erythrocytes instead of 500 erythrocytes. The deviations were considered to be of minor degree and to not compromise the validity of the study. Bone marrow toxicity, indicated by a reduced polychromatic to normochromatic erythrocyte ratio or signs of systemic toxicity, was not evident, but the test was performed at dose levels exceeding the limit concentrations specified in the current guideline. The study is therefore considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of male and female mice i*n vivo* under the conditions of this test. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.2.13. *In vivo studies in somatic cells – in vivo micronucleus, study 13*

Data point:	CA 5.4.2/013
Report author	[redacted]
Report year	1989
Report title	Mutagenicity study of glyphosate in NMRI mice using the micronucleus test
Report No	Not reported
Document No	Not reported
Guidelines followed in study	No guideline followed. The study was conducted according to the main criteria of OECD 474 (1983).
GLP	No, not conducted under GLP/ Officially recognised testing facilities. When the study was conducted, GLP was not compulsory.
Previous evaluation	Not accepted in RAR (2015)
Short description of study design and observations:	Glyphosate active ingredient (batch and purity not reported) was tested for induction of micronuclei in the bone marrow of NMRI mice. Based on the results of a preliminary toxicity study (data not shown), three groups of 7 mice/sex were treated with 2000 mg/kg bw glyphosate. The test substance was dissolved in water plus Tween 80 and administered as a single oral dose by gavage. In addition, groups of 5 mice/sex were included as control animals and received either the solvent (water plus Tween 80) by oral gavage or the positive control cyclophosphamide (100 mg/kg bw) by intraperitoneal injection. Glyphosate-treated animals were sacrificed 24, 48 and 72 hours after treatment and bone marrow slides were prepared. Sampling of the solvent control group was performed 48 hours after dosing and sampling of the positive control group was conducted 24 hours after treatment. Smears were prepared from the femoral bone marrow of each animal. About 1000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei. In addition, the amount of

	normochromatic erythrocytes (NCE) among 1000 PCE was determined.
Short description of results:	Mortality and clinical signs of toxicity were not reported. However, from the result tables it became obvious that 1/7 glyphosate-treated males of the 48-hour sampling group died during the course of the study. In addition, no results were scored for 1/7 glyphosate-treated males of the 72 hour sampling group. It was not specified why no scoring was performed for the animal, therefore it cannot be excluded that mortality has occurred. Without a dose-response relationship it can be assumed that if mortality occurred, it was not related to treatment. There was no significant increase in the number of NCE among 1000 PCE when compared to solvent controls for any sampling time point, indicating that no bone marrow toxicity was evident. In addition, there was no statistically significant increase in the frequency of micronucleated PCE (mPCE) or NCE at any sampling time point. A marked increase in the incidence of mPCE was observed for the positive control. Based on the experimental findings and under the conditions of the test, glyphosate active ingredient did not induce micronuclei in the bone marrow of male and female NMRI mice *in vivo*.
Reasons for why the study is not considered relevant/reliable or not considered as key study:	**Conclusion GRG:** The study was considered not acceptable, due to a large number of guideline deviations and reporting deficiencies. The test material purity and batch number were not indicated. According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study only 1000 polychromatic erythrocytes were evaluated. The guideline further recommends to include a negative/ vehicle control for every sampling time point or at least at the first sampling time point. In the present study the sampling of the solvent control animals was performed 48 hours after treatment, which represents the second sampling time point. Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not observed and systemic toxicity was not reported. In addition, mortality was not reported, however, from the result tables it became obvious that 1/7 glyphosate-treated males of the 48-hour sampling group died during the course of the study. In addition, no results were scored for 1/7 glyphosate-treated males of the 72 hour sampling group. It was not specified why no scoring was performed for the animal, therefore it cannot be excluded that mortality has occurred. In addition, evaluation criteria were not specified in the study report and no data on proficiency and/or historical control data were provided. The deviations were considered to compromise the validity of the study. Category 3b. **Conclusion AGG:** In line with the previous evaluation (RAR, 2015), the study is not considered acceptable for evaluation as described by GRG.

B.6.4.2.14. *In vivo studies in somatic cells – in vivo micronucleus, study 14*

Data point	CA 5.4.2/014
Report author	[redacted]
Report year	2009
Report title	Micronucleus test of Glyphosate TC in Bone Marrow Cells of the CD Rat by oral administration
Report No	23917
Document No	Not reported
Guidelines followed in study	OECD 474 (1997); Commission Directive 2000/32/EC B.12 (2000), USA EPA, JMAFF

Deviations from current test guideline OECD 474 (2016)	According to the current guideline OECD 474 (2016), at least 4000 polychromatic erythrocytes per animal should be evaluated for the presence of micronuclei. In the present study 2000 erythrocytes were evaluated (requirement of previous OECD guideline (1997)). Bone marrow exposure, indicated by a reduced polychromatic to normochromatic erythrocyte ratio, was not confirmed and there was no systemic toxicity observed, but dose levels included limit concentrations specified in the current guideline. In addition, historical control data were provided for solvent controls, but not for positive controls. The deviations were not expected to significantly impact the study outcome. It should, however, be noted that bone marrow exposure was not shown as there was no effect on the PCE/NCE ratio and no systemic toxicity was observed.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Valid, Category 2a **Conclusion AGG:** Some concerns were raised regarding the work conducted at this specific testing facility. After consultation with the responsible GLP monitoring authority, no GLP not in compliance (nic) reports on studies with glyphosate by this testing facility are available or known. The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

Glyphosate technical (batch: 20080801, purity: 98.8%) was tested for genotoxicity in CD rats using the micronucleus test. Based on the results of a preliminary toxicity study in which no signs of toxicity nor mortality were observed up to a dose level of 2000 mg/kg bw, 2000 mg/kg bw were selected as top dose for the main micronucleus study. Groups of 5 rats per sex were administered a single oral dose of 500, 1000 and 2000 mg glyphosate technical/kg bw dissolved in 0.8 % aqueous hydroxypropylmethyl cellulose at a constant dosage volume of 20 mL/kg bw. Vehicle control animals treated with 0.8 % aqueous hydroxypropylmethyl cellulose in an identical manner and positive control group animals receiving an intraperitoneal injection of 27 mg/kg bw cyclophosphamide were included in the experiment. All animals were observed for clinical signs of toxicity. Bone marrow sampling was performed 24 hours after treatment for all dose groups including the vehicle and positive controls and additionally after 48 hours for the control and high dose group. 2000 polychromatic erythrocytes (PCE) per animal were scored for the incidence of micronucleated PCE. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was determined for each animal by counting a total of 1000 erythrocytes.

Oral administration of glyphosate technical up to 2000 mg/kg bw did not induce any signs of systemic toxicity in the animals.

There was no difference in the ratio of PCE to NCE observed in test item treated rats when compared to the vehicle controls, indicating that no relevant cytotoxicity in bone marrow occurred. No increase in micronucleated PCE was observed in the treated groups when compared to the corresponding vehicle control group for any dose level and sampling time points. The positive reference item group exhibited a statistically significant increase in the number of micronucleated PCE, demonstrating the functionality of the test system.

Based on the results of the test and under the experimental conditions chosen, there is no evidence for the test item to induce clastogenicity/aneugenicity in rats *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate Technical

Identification: 37/064/08

Description: White solid

Lot/Batch #:	20080801
Purity:	98.8 % (w/w)
Stability of test compound:	The stability of the test item at storage conditions (at room temperature, in the dark) was guaranteed until the expiry date 01 Aug 2010. The stability of the test item in vehicle was not specified.
Solvent (vehicle) used:	0.8 % aqueous hydroxypropylmethyl cellulose

2. Control materials

Solvent (vehicle) control:	0.8 % aqueous hydroxypropylmethyl cellulose, 20 mL/kg bw administered by oral gavage
Positive control:	Cyclophosphamide, 27 mg/kg bw in 0.9 % NaCl, 20 mL/kg bw administered by intraperitoneal injection

3. Test animals:

Species:	Rat
Strain:	CD
Sex:	Males and females
Source:	[redacted]
Age at dosing:	32-33 days (males) and 33 - 34 days (females)
Weight at dosing:	106- 132 g (males) and 88 – 111 g (females)
Acclimation period:	At least 5 days
Diet/Food:	Commercial ssniff® R/M-H V1534 diet (ssniff Spezialdiäten GmbH, Soest, Germany), *ad libitum*, feeding was discontinued approx. 16 hours before administration
Water:	Tap water, *ad libitum*
Housing:	In groups of 2 - 3/sex in Makrolon type III plus cages with granulated textured wood bedding

4. Environmental conditions:

Temperature:	22 ± 3 °C
Humidity:	55 ± 15 %
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	500, 1000 and 2000 mg/kg bw
Dose volume:	20 mL/kg bw
Number of animals:	1/sex
Route of administration:	Oral gavage

b) Main micronucleus test

Dose levels:	500, 1000 and 2000 mg/kg bw
Dose volume:	20 mL/kg bw
Number of animals:	500 and 1000 mg/kg bw: 5/sex 2000 mg/kg bw: 10/sex
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 04 Feb – 06 Mar 2009
   Finalisation date: 18 May 2009

2. **Animal assignment and treatment:**

Preliminary toxicity study:
A preliminary study on acute oral toxicity was performed in male and female rats. One animal per sex received a single dose of 500, 1000 or 2000 mg/kg bw by oral gavage at a constant dosage volume of 20 mL/kg bw. The animals were observed for clinical signs of toxicity and mortality for a period of 3 days.

Main micronucleus test:
Based on the results of the preliminary toxicity study, the dose levels for the main micronucleus test were chosen. The top dose was defined as the dose producing signs of toxicity such that higher dose levels based on the same dosing regimen would be expected to produce lethality or as the dose which produces indications of bone marrow toxicity.

Groups of five rats each received a single oral dose of 500, 1000 and 2000 mg/kg bw glyphosate technical, which was administered at a constant dosage volume of 20 mL/kg bw. Similar constituted groups of control animals received the vehicle (0.8 % aqueous hydroxypropylmethyl cellulose) by oral gavage or the positive control (27 mg/kg bw in 0.9 % NaCl) by intraperitoneal injection. All animals were observed for clinical signs of toxicity before sacrifice.

Bone marrow sampling was performed 24 hours after treatment for all dose groups including the vehicle and positive control and additionally after 48 hours for the control and high dose group only.

3. **Slide preparation:**

After sacrifice, the femurs of the rats were excised and the bone marrow was flushed out with calf serum. The cells were centrifuged, re-suspended in a drop of calf serum and smears of 30 – 60 mm length were prepared. The smears were air-dried, fixed in methanol and stained with Mayers Haemaleum. Afterwards, the slides were rinsed with cold tap water and further stained with 0.5 % (w/v) ethanolic eosin. The slides were again left to air-dry, cleared in xylene and mounted.

4. **Slide evaluation:**

The slides were coded and randomized before microscopical analysis. 2000 polychromatic erythrocytes (PCE) per animal were scored for the incidence of micronuclei. In addition, the ratio of PCE to normochromatic erythrocytes (NCE) was determined for each animal by counting a total of 1000 erythrocytes.

5. **Statistics:**

The frequency of micronuclei and the ratio of PCE/NCE was analysed by comparing glyphosate treated samples to those of the positive and the solvent control. Statistical assessment was carried out using the chi-square test corrected for continuity. For each group, interindividual variation in the numbers of micronucleated PCE was estimated by means of a heterogeneity chi-square test. The numbers of micronucleated PCE in each treated group (males and females, separately and combined) were then compared with the numbers in the vehicle control groups by using a 2 x 2 contingency table to determine chi-square. Probability values of $p \leq 0.05$ were accepted as significant.

6. **Acceptance criteria:**

The micronucleus test was considered valid when the following criteria were met:

- The heterogeneity chi-square test provided evidence of acceptable variability between animals within a group.
- The incidence of micronucleated PCE in the vehicle control groups fell within or close to the historical vehicle control range of the laboratory.
- At least 7 animals (males and females together) out of each group at each kill were available for analysis.
- The positive reference chemical (CPA) induced clear and statistically significant increases in the frequencies of micronucleated PCE.

7. **Evaluation criteria:**

The test substance was considered as clearly positive if the following criteria were met:

- A statistically significant increase in the frequency of micronucleated PCE occurred for at least one dose at one sampling time.
- The frequency of micronucleated PCE at such a point exceeded the laboratory's historical control range.
- Corroborating evidence was obtained, for example, increased but statistically insignificant frequencies or micronucleated PCE at other doses or kill times, or dose response profiles.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in this study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

No signs of systemic toxicity or mortality were observed up to the limit dose of 2000 mg/kg bw. Based on the results of the preliminary toxicity study, the dose levels for the main study were selected to be 500, 1000 and 2000 mg/kg bw.

C. MAIN MICRONUCLEUS TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

There were no clinical signs of toxicity observed after administration of glyphosate technical up to the highest reasonable dose level of 2000 mg/kg bw until 48 hours after dosing.

Evaluation of bone marrow slides:

No difference in the ratio of PCE/NCE compared to the vehicle controls was observed in male and female rats treated with glyphosate technical, indicating that no relevant cytotoxicity in bone marrow occurred.

No test item-related increase in micronucleated PCE was observed in any treated group as compared to the corresponding vehicle control group at any of the two sampling time points.

The positive reference item group exhibited a statistically significant increase in the number of micronucleated PCE, demonstrating validity and sensitivity of the test system.

Table B.6.4.2.14-1: Micronucleus test of Glyphosate TC in Bone Marrow Cells of the CD Rat by oral administration [redacted], 2009), summary of genotoxicity data

Treatment	Dose (mg/kg bw)	Sampling time	Males		Females	
			mPCE ± SD /1000 PCE	PCE/NCE[$]	mPCE ± SD /1000 PCE	PCE/NCE[$]
HPMC	20 mL/kg	24 h	0.8 ± 0.6	0.65 ± 0.10	0.9 ± 0.2	0.65 ± 0.05
	20 mL/kg	48 h	1.0 ± 0.9	0.79 ± 0.20	1.1 ± 0.7	0.84 ± 0.11
HCD[#] mean			1.97	0.87	1.86	0.76
range			0.4 - 5.7	0.26 - 2.94	0.4 - 4.7	0.32 - 1.47
Test item	500	24 h	0.5 ± 0.6	0.65 ± 0.07	0.6 ± 0.7	0.65 ± 0.14
	1000	24 h	0.4 ± 0.2	0.77 ± 0.21	0.8 ± 0.4	0.56 ± 0.08
	2000	24 h	0.6 ± 0.4	0.72 ± 0.18	0.4 ± 0.4	0.59 ± 0.10
	2000	48 h	0.8 ± 0.4	0.98 ± 0.14	0.4 ± 0.4	0.74 ± 0.13
CPA	27	24 h	15.1 ± 5.2*	0.51 ± 0.05	12.0 ± 2.4*	0.55 ± 0.16

mPCE: micronucleated polychromatic erythrocytes, NCE: normochromatic erythrocytes

HPMC: 0.8 % hydroxypropylmethylcellulose, vehicle control

CPA: cyclophosphamide, positive control

[#] HCD Historical control data from the laboratory, average of group means from the most recent background data in 2009, data from 24, 48 and 72 h samplings combined

[$] per 1000 counted cells

* Statistical significance at $p \leq 0.05$ (chi square test)

III. CONCLUSION

Under the conditions of the test and based on the experimental results of the present study, glyphosate technical tested up to the limit dose of 2000 mg/kg bw did not show mutagenic properties in the rat bone marrow micronucleus test. Therefore, the test item was considered to be negative for clastogenicity/aneugenicity in rats.

Assessment and conclusion by applicant:

Negative for clastogenic/aneugenic effects in the bone marrow of CD rats *in vivo*.

The study was performed in compliance with GLP and in accordance with OECD guideline 474 (1997). There were only minor deviations when compared to the current OECD guideline 474 (2016). The number of polychromatic erythrocytes investigated per animal was 2000 (instead of 4000), which was the number recommended by the previous OECD guideline (1997). Bone marrow exposure, indicated by a reduced ratio of polychromatic to normochromatic erythrocytes, was not shown and no systemic toxicity was observed. However, test item doses included the limit dose of 2000 mg/kg bw. Therefore, the study was considered valid and acceptable.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for clastogenic/aneugenic effects in the bone marrow of CD mice i*n vivo* under the conditions of this test.
The study is considered acceptable but with restrictions (reliable with restrictions) due to the noted deviations.

B.6.4.2.15. *In vivo studies in somatic cells – in vivo chromosome aberration, study 1*

Data point	CA 5.4.2/015
Report author	[redacted]
Report year	1994
Report title	Genetic toxicology: *In vivo* mammalian bone marrow cytogenetic test – Chromosomal analysis
Report No	890-MUT-CH.AB
Document No	Not reported
Guidelines followed in study	OECD 475 (1984)
Deviations from current test guideline OECD 475 (2016)	Only 50 metaphase cells/ mouse were investigated for chromosomal aberrations, whereas the currently valid OECD guideline 475 (2016) suggests a total of 200 metaphase cells to be analysed for each animal. The mitotic index was determined for 100 cells instead of 1000 cells. Analysis of metaphase cells was only performed for control group animals and for animals of the high dose group, where toxicity was observed. There was no scoring for chromosome aberrations for lower dose levels where no toxicity occurred. The animals were treated twice, but the use of the second dose was not justified. The cell cycle arrest time was insufficient and the sampling time after the second dose was later (24 hours plus additional 1.5 hours cell cycle arrest) than specified in the current guideline (24 hours after 2nd dose including cell cycle arrest). Acceptance and evaluation criteria were not specified in the study report. Historical control data were not provided.
Previous evaluation	Yes, accepted in RAR (2015)

GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered supportive due to the noted deviations

Glyphosate (batch: 046, purity: 96.8 %) was investigated for its potential to induce chromosomal aberrations in bone marrow in male and female Swiss albino mice. The test item was dissolved in refined groundnut oil and administered by oral gavage. Based on the results of a preliminary toxicity study, in which no signs of systemic toxicity were noted at 5000 mg/kg bw/day, the same dose level was also chosen for the *in vivo* bone marrow chromosomal aberration study. Groups of 5 mice/sex received 50, 500 or 5000 mg/kg bw/day for two consecutive days at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of mice received the solvent or the positive control (50 mg/kg bw/day cyclophosphamide).

The animals were observed for clinical signs of toxicity and mortality twice a day and individual body weights were recorded on study Days 1, 2 and at sacrifice.

24 hours after the second treatment, the animals received an intraperitoneal injection of 0.4 % colchicine. 90 minutes following the injection of colchicine, the animals were sacrificed and bone marrow slides were prepared.
A total of 50 metaphases per mouse (250 per sex per condition) were scored for the presence of chromosomal aberrations and the number of dividing cells (mitotic index) was determined for 1000 cells per animal.

Upon treatment with glyphosate, 2/5 males of the high dose group were dull and had soft stool, which was considered to be incidental. In addition, there was a statistically significant reduction of body weight in females of the high dose group (26.8 ± 0.98 g instead of 32.2 ± 2.13 g in control animals). In animals of both sex, there was a statistically significant reduction in the mitotic index at 5000 mg/kg bw/day, indicating that bone marrow toxicity was evident.

There was no statistically significant and biologically relevant increase in the number of aberrant metaphases or in the incidence of individual chromosomal aberrations observed at the highest dose level of 5000 mg/kg bw/day when compared to solvent control animals. A statistically significant increase in the incidence of gaps in females was observed at 5000 mg/kg bw/day, however, the observations were considered to be without toxicological relevance. As the frequency of aberrant metaphase cells upon treatment with glyphosate was not increased at the highest dose level tested, the slides of the low and the mid dose groups were not investigated.

The frequency of aberrant metaphase cells in solvent control animals was as expected and the number of aberrant metaphases and incidences of aberration types was significantly high in cyclophosphamide treated animals. In line with significant toxicity in the bone marrow of cyclophosphamide treated animals, the results confirm the sensitivity of the animals to respond to chromosomal damaging agents.

Under the conditions of the test and based on the experimental findings, glyphosate did not increase the frequency of chromosomal aberrant metaphases in the bone marrow of male and female mice and was therefore considered negative for cytogenicity *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: Glyphosate (N-(Phosphonomethyl) glycine)

Identification: Not specified

Description: Odorless white crystals

Lot/Batch #: 046

Purity: 96.8 %

Stability of test compound:	The stability of the test item at storage conditions (at ambient temperature) was guaranteed until the expiry date July 1994. The stability of the test item in solvent was not specified. Dosing formulations were prepared fresh prior to treatment. Homogeneity of the test compound suspension was maintained by constant stirring/mixing in mortar during treatment.
Solvent (vehicle) used:	Refined groundnut (peanut) oil

2. Control materials

Solvent (vehicle) control:	Refined groundnut (peanut) oil
Positive control:	Cyclophosphamide (Endoxan-ASTA), 50 mg/kg bw/day

3. Test animals:

Species:	Mouse
Strain:	Swiss albino
Sex:	Male and female
Source:	[redacted]
Age at dosing:	8 - 12 weeks
Weight at dosing:	32 – 38 g (males) and 28 – 32 g (females)
Acclimation period:	At least one week
Fasting period prior administration:	Gold Mohur pelleted mice feed (Lipton India Ltd., Bangalore, India), *ad libitum*
Diet/Food:	Protected, deep borewell water, passed through activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter-cum-purifier, *ad libitum*
Water:	In groups of 5/sex in sterilised standard polypropylene cages measuring 290 x 220 x 140 mm with paddy husk bedding
Housing:	Mouse

4. Environmental conditions:

Temperature:	22 ± 3 °C
Humidity:	40 - 70 %
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

a) Preliminary toxicity study

Dose levels:	2000 and 3000 mg/kg bw/day
Concentrations:	Not specified
Dose volume:	Not specified
Number of animals:	2/sex/group
Route of administration:	Oral gavage

b) Main cytogenicity test

Dose levels:	50, 500 and 5000 mg/kg bw/day
Concentrations:	5.1, 51.5 and 334.0 mg/mL
Dose volume:	10 mL/kg bw (low and mid dose), 15 mL/kg bw (high dose)
Number of animals:	5/sex/group
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. **Dates of experimental work:** 11 Jan – 09 Feb 1993
   Finalisation date: 22 Jan 1994

2. **Animal assignment and treatment:**

Preliminary toxicity study:
In a range-finding study two male and two female mice were administered dose levels of 2000 and 3000 mg/kg bw/day for two consecutive days to determine the maximum tolerated dose (MTD). Based on the results of the preliminary toxicity study, dose levels for the bone marrow chromosomal aberration study were chosen.

Main micronucleus test:
Groups of five mice per sex were administered for two consecutive days at doses of 50, 500 and 5000 mg/kg bw/day. The test item was dissolved in refined groundnut (peanut) oil and administered via oral gavage at a constant dosage volume of 10 mL/kg bw. Similar constituted groups of 5 mice/sex were treated with the solvent or positive control (50 mg/kg bw/day cyclophosphamide).
The animals were observed for clinical signs of toxicity and mortality twice a day and individual body weights were recorded on study Days 1, 2 and at sacrifice.

24 hours after the second treatment, the animals received an intraperitoneal injection of 0.4 % colchicine at a dosage volume of 10 mL/kg bw. 90 minutes later, the animals were sacrificed by cervical dislocation and bone marrow slides were prepared.

3. **Slide preparation:**

After sacrifice, the femur bones from both sides were removed, dissected and bone marrow cells were flushed out with 0.56 % potassium chloride solution. The cell suspensions underwent hypotonic treatment at 37 °C for 15 minutes, followed by centrifugation and drop-wise re-suspension in freshly prepared methanol and glacial acetic acid (3:1) fixative. The cells were fixed two times for 10 minutes, followed by 1 hour fixation in the refrigerator. The cell suspension was dropped onto a clean chilled slide and flame dried. Staining was performed with 2 % Giemsa for 10 minutes, afterwards the slides were rinsed with distilled water, blow dried, immersed in xylene and coverslip mounted with DPX.

4. **Metaphase analysis:**

Slides were coded and 50 metaphase cells per animal (250 per condition) were scored for chromosomal aberrations. Number and frequency of metaphase cells were recorded and evaluated for aberrations such chromatid or chromosome gaps, breaks, acentric fragments, ring chromosomes, multiple chromatid breaks, pulverization, polyploidy and exchange figures.

In addition, toxicity was evaluated based on the mitotic index, representing the frequency of metaphase cells in 100 cells per slide.

5. **Statistics:**

Intra-group body weight changes during the treatment period were compared by paired t-test. Chromosomal aberrations in treated and positive control animals versus solvent control animals were statistically analysed by Z-test.

6. **Acceptance criteria:**

Acceptance criteria were not specified in the study report.

7. **Evaluation criteria:**

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not compulsory by the test guideline.

B. PRELIMINARY TOXICITY STUDY

There were no clinical signs of toxicity and no changes in body weight observed. The maximum tolerated dose (MTD) was determined to be > 3000 mg/kg bw/day. Based on the results of the dose-range finding study, the dose levels for the bone marrow cytogenicity study were selected.

C. BONE MARROW CHROMOSOMAL ABERRATION TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

There were no treatment-related signs of systemic toxicity at 50 and 500 mg/kg bw/day. In the high dose group, 2/5 males were dull and had soft stool. The findings were considered toxicologically not relevant.

Body weight:

At sacrifice, there was a statistically significant reduction in body weight development of females of the high dose group. The observation was attributed to treatment and considered toxicologically relevant.

Table B.6.4.2.15-1: Genetic toxicology: *In vivo* mammalian bone marrow cytogenetic test – Chromosomal analysis ([redacted] 1994), body weight changes

Group	Dose [mg/kg bw/day]	Males			Females		
		Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Solvent		35.2 ± 2.04	35.6 ± 1.49	36.0 ± 1.79	28.8 ± 0.98	29.2 ± 0.98	26.8 ± 1.60
Test item	50	36.0 ± 1.26	35.6 ± 0.80	35.2 ± 1.60	28.4 ± 0.80	27.6 ± 1.96	26.4 ± 1.96
	500	34.8 ± 1.60	34.0 ± 1.79	32.3 ± 2.13	29.6 ± 0.80	28.8 ± 0.98	29.2 ± 0.98
	5000	34.8 ± 1.60	36.0 ± 1.26	35.2 ± 1.60	30.8 ± 0.98	28.8 ± 1.60	26.8 ± 0.98*
CPA	50	36.8 ± 1.60	36.8 ± 1.60	35.4 ± 2.30	29.6 ± 1.49	29.6 ± 1.49	29.2 ± 1.60

CPA: Cyclophosphamide, positive control

* Statistically significantly decreased compared to control by paired t-test.

Evaluation of bone marrow slides:

There was no statistically significant and biologically relevant increase in the number of aberrant metaphases or in the incidence of individual chromosomal aberrations observed at the highest dose level of 5000 mg/kg bw/day when compared to solvent control animals.

According to the applicant's summary, a statistically significant increase in the incidence of gaps in females was observed at 5000 mg/kg bw/day. **Note RMS:** This is in disagreement with the study report where no significant increase in the incidence of gaps in females was seen.

In both, males and females of the 5000 mg/kg bw/day group there was once incidence of each chromosome exchange figure (statistically not significant). The observations were both considered to be without toxicological relevance.

In addition, there was a statistically significant reduction in the mitotic index at 5000 mg/kg bw/day, indicating that bone marrow toxicity was evident.

As the frequency of aberrant metaphase cells upon treatment with glyphosate was not increased at the highest dose level tested, the slides of the low and the mid dose groups were not investigated.

The frequency of aberrant metaphase cells in solvent control animals was as expected and the number of aberrant metaphases and incidences of aberration types was significantly high in cyclophosphamide treated animals. In line with significant toxicity in the bone marrow of cyclophosphamide treated animals, the results confirm the sensitivity of the animals to respond to chromosomal damaging agents.

Table B.6.4.2.15-1: Genetic toxicology: *In vivo* mammalian bone marrow cytogenetic test – Chromosomal analysis ([redacted], 1994), summary of genotoxicity data in rats

Compound	Dose [mg/kg bw/day]	Sampling time point	Number of animals (♂ + ♀)	No. of metaphases scored	Genotoxicity						Mitotic index [%]
					No. structural aberrant cells		% structural aberrant cells		PI	Judge	
					incl. gaps	**excl. Gaps**	incl. gaps	**excl. gaps**	No. polyploid cells		
Males											
Solvent (HBSS)	/	24 h	5	250	18	**12**	7.20	**4.80**	0	negative	13.34
Test item	5000	24 h	5	250	15	**10**	6.00	**4.00**	1	negative	8.87*
Positive control (CPA)	50	24 h	5	250	164*	**139***	65.5*	**55.6***	2	positive	14.68
Females											
Solvent (HBSS)	/	24 h	5	250	15	**10**	6.00	**4.00**	0	negative	17.42
Test item	5000	24 h	5	250	27	**11**	10.80	**4.40**	1	negative	9.54*
Positive control (CPA)	50	24 h	5	250	171*	**155***	68.4*	**62***	0	positive	5.53*
Both sexes combined											
Solvent (HBSS)	/	24 h	10	500	33	**22**	13.20	**8.80**	0	negative	15.30
Test item	5000	24 h	10	500	42	**21**	16.80	**8.40**	2	negative	9.20*
Positive control (CPA)	50	24 h	10	501	335*	**294***	134*	**117.6***	2	positive	10.10*

CPA: Cyclophosphamide

*: Statistically significantly when compared to control by "Z" test

PI: polyploid cells

III. CONCLUSION:

Based on the results of the present study, glyphosate did not increase the frequency of chromosomal aberrant metaphases in the bone marrow of male and female mice. Thus, the test item was considered negative for cytogenicity *in vivo* under the conditions of the test.

Assessment and conclusion by applicant:

Negative for chromosome aberrations in the bone marrow of male and female Swiss albino mice *in vivo*.

The study was performed in compliance with GLP and according to OECD guideline 475 (1984). When compared to the current OECD guideline 475 (2016), a number of deviations became evident. The number of metaphase cells scored for chromosome aberrations was 50, whereas 200 metaphases should be evaluated according to the current guideline. Likewise, the mitotic index was determined for 100 cells instead of 1000 cells. The animals were treated twice, but the use of the second dose was not justified.

Bone marrow exposure was indicated by a reduction in the mitotic index and clinical signs of toxicity and impaired body weight analysis were observed in animals of the high dose group. Analysis of metaphase cells was only performed for control group animals and for animals of the high dose group, were toxicity was observed. There was no scoring for chromosome aberrations for lower dose levels where no toxicity occurred. However, as no induction of chromosomal aberrations was observed at this concentration, it can be assumed that the number of chromosome aberrations was comparatively low at lower test item doses. Further deviations to the current guideline were considered to be of minor degree. Due to the large number of deviations and because only a very low number of metaphase cells was investigated per animal, the study was considered to provide supporting information.

Evidence of bone marrow exposure was used to read across to micronucleus assays in mice of the same strain reported by the same study authors.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for chromosome aberrations in the bone marrow of male and female Swiss albino mice *in vivo* under the conditions of this test. According to the RMS a statistically significant increase in the incidence of gaps in females was not observed at 5000 mg/kg bw/day in contrast to the description in the above summary. The study is considered supportive due to the noted deviations.

B.6.4.2.16. *In vivo studies in somatic cells – in vivo chromosome aberration study 2*

Data point	CA 5.4.2/016
Report author	[redacted]
Report year	1983
Report title	*In Vivo* Bone Marrow Cytogenetics Study of Glyphosate in Sprague-Dawley Rats
Report No	830083
Document No	M-645019-01-1
Guidelines followed in study	No guideline followed, study was conducted similar to OECD 475 (2016)
Deviations from current test guideline OECD 475 (2016)	The test item was administered by intraperitoneal injection, which does not represent an intended route for human exposure. In addition, the test was conducted as limit test with a dose level of 1000 mg/kg bw, whereas the current OECD TG 475 suggests a dose level of 2000 mg/kg bw. There was no evidence that the test item was administered at a dose level which induces toxicity, as no clinical signs of systemic toxicity were observed and no bone marrow toxicity became evident. Furthermore, no body weight data or individual toxicity data were reported. In addition, the mitotic index as an indicator for cytotoxicity was not calculated and the number of polyploid cells was not determined. A total of 50 metaphase cells/rat were investigated for chromosomal aberrations, whereas the currently valid OECD guideline 475 (2016) suggests that at least 200 metaphases should be analysed for each animal. In addition, sampling time points for bone marrow cells was 6, 12 and 24 hours, but a latter sampling time point (24 hours after the first sampling time point) was not included. The weight of the animals was not reported. In addition, acceptance and evaluation criteria were not specified in the study report. Historical control data were not provided.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, not conducted under GLP /Officially recognised testing facilities. When the study was performed, GLP was not compulsory.
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered supportive due to the noted deviations.

A mammalian bone marrow chromosomal aberration test was performed in male and female Sprague-Dawley rats in order to investigate clastogenic effects of glyphosate (batch: T830044, purity: 98.7%) *in vivo*.

The test substance was dissolved in Hank's Buffered Salt Solution (HBSS) and administered to 3 groups of fasted animals via intraperitoneal injection. All three groups of 6 rats per sex received a test item dose of 1000 mg/kg bw. Similarly constituted groups of rats received the vehicle (HBSS) or the positive control cyclophosphamide and served as controls. About 4, 10 and 22 hours following treatment, each one group of test item and solvent control animals was injected with 2 mg/kg bw colchicine. Two hours after colchicine administration (6, 12 and 24 hours after treatment), the animals were sacrificed and bone marrow slides were prepared for each animal. Bone marrow samples for the positive control group animals were prepared from the 24 hour sampling time only.

A total of 50 metaphases per animal (300 per sex per condition) were scored for the presence of chromosomal aberrations.

Clinical signs of systemic toxicity were not reported for either control or glyphosate treated animals and no signs of bone marrow toxicity became evident.

A significant increase in the number of aberrant metaphases was not observed for any of the three sampling time points upon treatment with glyphosate. The type and frequency of any chromosomal aberrations in glyphosate-treated rats was comparable to those of solvent control animals.

In contrast, rats treated with the positive control cyclophosphamide showed a strong increase in the frequency of structural chromosome aberrations about 24 hours after treatment. In addition, a reduction in metaphase cells was observed for these animals, indicating that bone marrow toxicity was evident. As solvent and positive control animals showed the expected results, the sensitivity of the test system and the capability of the animals to respond to clastogenic substances was demonstrated.

Based on the results of the experiment and under the tested conditions, glyphosate was considered negative for clastogenicity in male and female rats *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

Identification:	Glyphosate
Description:	White powder
Lot/Batch #:	T830044
Purity:	98.7 %
Stability of test compound:	The stability of the test item was not reported. Storage at room temperature was recommended. The stability of the test item in solvent was not specified.
Solvent (vehicle) used:	Hank's Buffered Salt Solution (HBSS)

2. Control materials

Solvent (vehicle) control:	Hank's Buffered Salt Solution (HBSS)
Positive control:	Cyclophosphamide, 25 mg/kg bw in HBSS

3. Test animals:

Species:	Rat
Strain:	Sprague-Dawley (Crl: CD®(SD)BR)
Sex:	Male and female

Source:	[redacted]
Age at dosing:	Approx. 9 weeks
Weight at dosing:	Not specified
Acclimation period:	Approx. 7 days
Fasting period prior administration:	14 – 24 hours
Diet/Food:	Purina Laboratory Rodent Chow® No. 5002 (Ralston-Purina Company, St. Louis, Missouri, USA), *ad libitum*
Water:	Tap water supplied by the public water system, *ad libitum*
Housing:	In stainless steel mesh cages suspended over absorbent paper bedding

4. Environmental conditions:

Temperature:	21 - 23°C
Humidity:	35 -60 %
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

Dose levels:	1000 mg/kg bw
Concentrations:	100 mg/mL
Dose volume:	10 mL/kg bw
Number of animals:	6 males and 6 females
Route of administration:	Intraperitoneal injection

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 08 – 12 Aug 1983
Finalisation date: 20 Oct 1983

2. Animal assignment and treatment:

Three groups of 6 rats per sex (12 rats/ group in total) received an intraperitoneal injection of 1000 mg/kg bw glyphosate, which was administered at a constant dosage volume of 10 mL/kg bw. Prior to treatment, the animals were fasted for 14-24 hours. Similar constituted groups of control animals received the vehicle (HBSS) or the positive control (25 mg/kg bw cyclophosphamide in HBSS).

About 4, 10 and 22 hours following treatment, each one group of test item and solvent control animals was injected with 2 mg/kg bw colchicine. Two hours after colchicine administration (6, 12 and 24 hours after treatment), the animals of the test item and the solvent control group were sacrificed by CO_2 asphyxiation and severance of the spinal cord. Bone marrow slides from the positive control group were sampled 24 hours after treatment only.

3. Slide preparation:

Immediately after sacrifice, both femurs of the animals were dissected and marrow was aspirated from each femur and mixed with HBSS. The cells were pelleted by centrifugation and re-suspended in 0.075 M potassium chloride. After removal of gross debris, the cells were incubated in a water bath at 37 °C for 30 minutes, and subsequently fixed with Cornoy's fixative (methanol/glacial acetic acid 3:1 v/v). The cells were centrifuged again, re-suspended in fixative, dropped onto clean wet slides and flamed to facilitate spreading of chromosomes. The slides were air-dried and stained with 2 % Giemsa solution for 15-20 minutes, rinsed with water, air-dried again and covered with cover slips.

4. Metaphase analysis:

Slides were randomly coded and approximately 50 mitotic cells per animal (300 per condition) were scored for chromosomal aberrations. Number and frequency of metaphase cells were recorded and evaluated for structural chromosomal aberrations such as dicentric chromosomes, ring chromosomes, chromosome deletions, gaps. In addition, chromatid aberrations and the number of aneuploidy cells were recorded.

5. Statistics:

The student's t-test was used for data analysis.

6. Acceptance criteria:

Acceptance criteria were not specified in the study report.

7. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed.

B. BONE MARROW CHROMOSOMAL ABERRATION TEST

Systemic toxicity:

Mortality:

No mortality occurred.

Clinical signs of toxicity:

No clinical signs of systemic toxicity were reported.

Evaluation of bone marrow slides:

A significant increase in the number of aberrant metaphases was not observed for any of the three sampling time points upon treatment with glyphosate. Although a small number of chromatid aberrations was noted 6, 12 and 24 hours after treatment, the frequency was comparable for glyphosate-treated and solvent control animals. In addition, there was no evidence for bone marrow toxicity in any of the test item treated groups.

A huge increase in the frequency of chromosomal aberrant cells, accompanied with strong evidence for bone marrow toxicity was observed for the positive control cyclophosphamide 24 hours after treatment, demonstrating the animal's capability to respond to clastogenic substances and confirming the sensitivity of the test.

Table B.6.4.2.16-1: *In Vivo* Bone Marrow Cytogenetics Study of Glyphosate in Sprague-Dawley Rats ([redacted] 1983), summary of genotoxicity data

Compound	Dose [mg/kg bw]	Sampling time point	Number of animals (♂ + ♀)	No. of metaphases scored	Genotoxicity							
					No. structural aberrant cells	% structural aberrant cells	No. of aberrations		Rel. No. of structural aberrations §		Numerical aberrations	Judge
					incl. gaps#	incl. gaps	incl. gaps	**excl. gaps**	incl. gaps	**excl. Gaps**	No. aneugenic cells$	
Solvent (HBSS)	/	6 h	12	600	12	2.00	13	8	2.17	1.33	35	Negative
		12 h	12	575	7	1.22	5	2	0.87	0.35	48	Negative

Table B.6.4.2.16-1: *In Vivo* Bone Marrow Cytogenetics Study of Glyphosate in Sprague-Dawley Rats ([redacted] 1983), summary of genotoxicity data

Compound	Dose [mg/kg bw]	Sampling time point	Number of animals (♂ + ♀)	No. of metaphases scored	Genotoxicity							
					No. structural aberrant cells	% structural aberrant cells	No. of aberrations		Rel. No. of structural aberrations §		Numerical aberrations	Judge
					incl. gaps#	incl. gaps	incl. gaps	**excl. gaps**	incl. gaps	**excl. Gaps**	No. aneugenic cells$	
		24 h	12	565	10	1.77	12	4	2.12	0.71	46	Negative
Test item	1000	6 h	12	600	16	2.67	18	6	3.00	1.00	43	Negative
		12 h	12	577	13	2.25	14	5	2.43	0.87	42	Negative
		24 h	12	492	13	2.64	13	7	2.64	1.42	35	Negative
Positive control (CP)	25	24 h	12	277	113	40.79	351	314	126.71	113.36	23	Positive

CP: Cyclophosphamide

#: Calculated based on raw data from study report as follows: no of cells scored - no of normal cells (incl. aneuploid cells)

$: Almost all aneuploid cells were minus one chromosome, and since slides had been flamed, these cells were considered to be technical artefacts

§: in 100 metaphases, calculated as follows: = (100/number of metaphases scored) x number of aberrations

III. CONCLUSION

Based on the results of the present study, glyphosate did not increase the frequency of chromosomal aberrant metaphases in the bone marrow of male and female rats. Thus, the test item was considered negative for clastogenicity in vivo under the conditions of the test.

3. Assessment and conclusion

Assessment and conclusion by applicant:

Negative for chromosome aberrations in the bone marrow of male and female Sprague-Dawley rats *in vivo*.

The study was conducted not compliant with GLP but similar to OECD guideline 475 (2016). However, a number of deviations became evident. The test item was administered by intraperitoneal injection, which does not represent an intended route for human exposure. In addition, the test was conducted as limit test with a dose level of 1000 mg/kg bw, whereas the current OECD TG 475 suggests a higher dose level of 2000 mg/kg bw. There was no evidence that the test item was administered at a dose level which induced toxicity, as no clinical signs of systemic toxicity were observed and no bone marrow toxicity became evident. However, due to the route of exposure selected (intraperitoneal injection) it can be assumed that the bone marrow was exposed. In addition, the mitotic index as an indicator for cytotoxicity was not calculated and the number of polyploid cells was not determined. A total of 50 metaphase cells/rat were investigated for chromosomal aberrations, whereas the currently valid OECD guideline 475 (2016) suggests that at least 200 metaphases should be analysed for each animal.

Further deviations were considered to be of minor degree. Due to the large number of guideline deviations, the study was considered to provide supporting information.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for chromosome aberrations in the bone marrow of male and female Sprague-Dawley rats *in vivo* under the conditions of this test. The study is considered supportive due to the noted deviations.

B.6.4.3. In vivo studies in germ cells

B.6.4.3.1. *In vivo study in germ cells – Dominant lethal assay, study 1*

Data point	CA 5.4.3/001, CA 5.4.3/002 and CA 5.4.3/003
Report author	[redacted]
Report year	1992
Report title	Dominant lethal test in Wistar rats
Report No	TOXI- 888-DLT
Document No	Not reported
Guidelines followed in study	OECD 478 (1984)
Deviations from current test guideline OECD 478 (2016)	Dose levels were spaced with a factor of 5, which is above the factors (2 – 4) specified in the current OECD guideline 478 (2016). Necropsy was performed on Day 16 after pairing rather than on gestation Day 16. The number of implantations per group for each mating period was below the recommended number of 400 implants per group as required according to OECD 478 (2016). Foetal body weights were not recorded. No information on historical control data was provided. There was no raw data on individual animals treated with the positive control provided. The mean pre- and post-implantation losses per dam were not calculated, but percentages of pre-implantation losses, as well as percentages of post-implantation losses (corresponding to the dominant lethal factor) were reported. Acceptance and evaluation criteria were not specified in the study report. Statistical assessment of under- and overdispersion was not performed.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered supportive due to the noted deviations.

Glyphosate (code FSG 03090 H/05, March 1990, cas nr.: 1071-83-6, purity: 96.8 %) was investigated in a dominant lethal study in Wistar rats. The test item was dissolved in refined groundnut (peanut) oil and administered by oral gavage to groups of 30 males at dose levels of 200, 1000 and 5000 mg/kg bw (single dose) and a constant dosage volume of 10 mL/kg bw. A similar constituted group of males received the solvent control. Two positive control groups of 5 males received ethylmethanesulphonate either administered at a treatment regime of 100 mg/kg bw/day for 5 consecutive days or as single dose of 500 mg/kg bw.

Immediately after dosing, the males were paired with untreated virgin females (mating ratio 1:1) for 6 days. On the 8th day after treatment, the males were again paired with a new batch of virgin females. The procedure was repeated for 10 consecutive weeks. All animals were observed twice daily for clinical signs. Body weights were recorded for males prior to treatment, on Days 1, 2, 4 and 6 and weekly thereafter. Females were weighed prior to mating and at terminal sacrifice. The females from each mating interval were sacrificed on gestation Day 16. The uterus and ovaries were dissected and investigated for the number of *corpora lutea*, implantations, early resorptions, fetal and embryonic resorptions and the number of live implants. All males were subjected to gross necropsy following the last pairing.

Oral treatment with glyphosate did not induce mortality in any animal. Clinical signs of toxicity were observed predominantly for animals of the high dose group throughout the whole study period and comprised nasal discharge, rough hair coat, snuffling, soft stool/diarrhea and urine incontinence. In addition, the body weight of sires was decreased in the 1000 and 5000 mg/kg bw dose group during the first four days of treatment. Both observations were attributed to treatment and considered toxicologically relevant.

At terminal necropsy, unilateral testicular atrophy was observed in 3/30 males of the high dose group. Male fertility was not impaired upon treatment with glyphosate.

Investigation of the female uteri contents revealed an acute toxic effect of glyphosate at a dose level of 5000 mg/kg bw after the first mating. The number and percentage of pregnant females and the number of implantations per dam were significantly lower than for control animals. In line with these findings, there was an increased incidence of early resorptions and of pre- and post-implantation losses in the animals of this group. In the first mating group, there were no adverse effects on the number of *corpora lutea*, the number of fetal and embryonic resorptions and the number of live implants per dam.

Fertility indices during the remaining 9 study weeks were not considered to be affected by treatment. Although changes of statistical significance were observed for the number of implantations, the number of early, fetal and embryonic resorptions, the number of live implants and the number of pre- and post-implantation losses, these fluctuations and highly variable values for dominant lethality were observed among all dose groups and without any relation to dose or duration of treatment.

Treatment with the positive control ethylmethanesulphonate had statistically significant dominant lethal effects for both treatment regimes. A significant decrease in the incidence of pregnancy to 0 % was observed in the third mating interval and a gradual recovery back to normal by 9 to 10 weeks. In addition, there was an increased incidence of small moles /fetal resorptions during the first week, which gradually recovered to normal by week 9.

Based on the experimental findings and under the conditions of the test, treatment of males with glyphosate was considered to induce acute toxic effects at 5000 mg/kg bw in male and female animals during the first mating period. Under the conditions of the test, glyphosate did not induce dominant lethal effects in Wistar rats and was therefore considered non-genotoxic to germ cells *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

Identification:	Glyphosate
Description:	Odourless, white crystals
Lot/Batch #:	Code FSG 03090 H/05, March 1990
Purity:	96.8 %
Stability of test compound:	Under storage conditions (at ambient temperature) stability was guaranteed for more than two years. Dosing formulations were prepared fresh prior to treatment.
Solvent (vehicle) used:	Refined groundnut (peanut) oil

2. Control materials

Solvent (vehicle) control:	Refined groundnut (peanut) oil
Positive control:	Ethylmethanesulphonate, 100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water

3. Test animals:

Species:	Rat
Strain:	Wistar
Sex:	Males and females
Source:	Bred at [redacted]
Age at study initiation:	20 – 24 weeks
Weight at study initiation:	> 250 g (males) and > 150 g (females)
Acclimation period:	At least 10 days
Diet/Food:	Pelleted rat feed (M/S Lipton India Ltd., Bangalore, India), *ad libitum*
Water:	Deep borewell water, passed through activated charcoal filter and exposed to UV rays in Aquaguard on-line water filter-cum-purifier, *ad libitum*
Housing:	Before treatment: In groups of 15-20/sex in suspended stainless steel wire mesh cages with clean paddy husk bedding. During mating: On a 1:1 basis (male:female) in a standard polypropylene cages measuring 290 x 220 x 140 mm) with sterilized paddy husk bedding. Post mating: Females were housed in groups of 5 in serial order from the same group in standard polypropylene cages measuring 430 x 270 x 150 mm) with sterilized paddy husk bedding.

4. Environmental conditions:

Temperature:	22 ± 3 °C
Humidity:	52 - 70 %
Air changes:	Approximately 10 - 15/hour
Photoperiod:	12-hour light and dark cycle

5. Test concentrations and treatment groups:

Dose levels:	200, 1000 and 5000 mg/kg bw
Dose volume:	10 mL/kg bw
Number of animals:	30 males and 300 females per group (30 females per mating interval)
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 03 Sep 1991 – 31 Jan 1992
Finalisation date: 04 Nov 1992

2. Animal assignment and treatment:

Groups of 30 males per dose received a single administration of glyphosate by oral gavage at dose levels of 200, 1000 and 5000 mg/kg bw at a constant dosage volume of 10 mL/kg bw. Similar constituted groups received the solvent (refined groundnut oil) or the positive control ethylmethane sulfonate. The positive control substance was dissolved in distilled water and administered to two groups of 5 males at a treatment regimen of 100 mg/kg bw/day for 5 consecutive days and as single dose of 500 mg/kg bw.

Immediately after dosing, the males were paired with untreated virgin females (mating ratio 1:1) for 6 days. On Day 8 after treatment, the males were again paired with a new batch of virgin females. The procedure was repeated for 10 consecutive weeks. The females from each mating interval were sacrificed on gestation Day 16.

3. Observations

All animals were observed twice daily during the first week and once daily during the remaining period for signs of toxicity and mortality. The time of onset, degree and duration of clinical signs was recorded.

4. Body weight

The body weight of male animals was recorded prior to treatment, on Days 1, 2, 4 and 6 and weekly thereafter. For females, the initial body weight and the body weight at terminal sacrifice were noted.

5. Sacrifice

All animals were sacrificed under ether anaesthesia.
Males were sacrificed after 10 mating intervals and investigated for gross necropsy changes in the visceral organs. From all sires the following organs were collected and preserved in 10 % neutral buffered formalin: testes, epididymides, seminal vesicles and prostate.

Females from each mating interval were sacrificed on gestation Day 16. The uterus along with the ovaries was dissected and the following parameters were recorded prior to preservation in 10 % neutral buffered formalin:

- Number of *corpora lutea* in each ovary
- Number of implantations
- Number of early resorptions
- Number and percentage of small moles/embryonic resorptions
- Number and percentage of large moles/fetal resorptions
- Number and percentage of live implants

Furthermore, the percentage of pre- and post-implantation losses were determined according to the following formulas:

$$\text{Pre} - \text{implantation loss } [\%] = \frac{\text{Total number of corpora lutea} - \text{Number of implantations}}{\text{Total number of corpora lutea}} \times 100$$

$$\text{Post} - \text{implantation loss } [\%] = \frac{\text{Number of early} + \text{late embryonic deaths}}{\text{Total number of implantations}} \times 100$$

The percentage of post-implantation losses corresponds to the dominant lethal factor.

6. Statistics:

The weekly male body weight, the number of *corpora lutea*, the number of implantations, the number of early resorptions, the number and percentage of small and large moles, the number and percentage of live implants and the percentage of pre- and post-implantation losses were statistically analysed using the Bartlett's test for homogeneity, analysis of variance and Dunnett's multiple pairwise comparison. The number of early resorptions were compared by Mann Whitney test; pregnancy indices were analysed by "Z"-test. Results of the statistical analysis were designated in comparison to the solvent control group.

7. Acceptance criteria:

Acceptance criteria were not specified in the study report.

8. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in this study, as not required by the test guideline.

B. OBSERVATIONS

Systemic toxicity:

Mortality:

One sire of the positive control group in the second dose regime (single treatment with 500 mg/kg bw) died during study week 4. There were no deaths in the dose groups treated with glyphosate.

Clinical signs of toxicity:
Clinical signs of toxicity were observed among all treatment and control groups. Commonly and frequently observed symptoms included nasal discharge, rough hair coat, snuffling, soft stool/diarrhea and urine incontinence. The incidence was increased at 5000 mg/kg bw, which was attributed to treatment and considered toxicologically relevant. In animals of the high dose group, the clinical signs were observed throughout the entire study period.

Table B.6.4.3.1-1: Dominant lethal test in Wistar rats ([redacted] 1992), clinical signs of toxicity observed in sires

Clinical sign of toxicity	Dose group [mg/kg bw]			
	0	200	1000	5000
Nasal discharge	1/30	0/30	2/30	9/30
Rough hair coat	0/30	0/30	0/30	4/30
Snuffling	3/30	0/30	0/30	10/30
Soft stool / diarrhea	1/30	0/30	1/30	5/30
Urine incontinence	0/30	0/30	5/30	1/30

C. BODY WEIGHT AND BODY WEIGHT DEVELOPMENT OF SIRES

Starting body weights of all glyphosate treated groups were statistically significantly higher than those of the solvent control group. The difference continued during the entire study period until final sacrifice. Upon treatment with the test item, body weight of sires was decreased in the 1000 and 5000 mg/kg bw group during the first four days of treatment, but was comparable to those of solvent control treated animals thereafter. The observation was considered treatment-related.

Table B.6.4.3.1-2: Dominant lethal test in Wistar rats ([redacted] 1992), body weights in sires

		Dose group [mg/kg bw]			
		0	200	1000	5000
Days in study week 1	**0**	270 ± 26	303 ± 11*	329 ± 34*	314 ± 32*
	1	285 ± 26	305 ± 13*	317 ± 34*	303 ± 32*
	2	292 ± 25	310 ± 14*	323 ± 34*	297 ± 30*
	4	297 ± 26	313 ± 14*	325 ± 32*	308 ± 32*
	6	304 ± 26	321 ± 15*	332 ± 32*	316 ± 31*
Study week no.	**2**	305 ± 25	320 ± 15*	332 ± 32*	317 ± 32*
	3	322 ± 27	330 ± 18*	350 ± 30*	333 ± 34*
	4	331 ± 32	341 ± 20	350 ± 33	334 ± 33
	5	331 ± 32	344 ± 29*	359 ± 32*	352 ± 35*
	6	342 ± 34	348 ± 25	363 ± 33	352 ± 39
	7	350 ± 34	358 ± 29	360 ± 32	358 ± 33
	8	346 ± 34	362 ± 28*	374 ± 31*	364 ± 35*
	9	351 ± 35	359 ± 30*	376 ± 31*	369 ± 39*
	10	339 ± 43	357 ± 34*	376 ± 34*	371 ± 39*
Sacrifice		347 ± 38	359 ± 33*	388 ± 34*	371 ± 40*

* Statistically significant difference from control group by Dunnett's test

D. NECROPSY OF SIRES

At terminal sacrifice, unilateral testicular atrophy was noted in 3/30 sires of the high dose group. The effect was considered treatment-related. There were no further gross lesions in visceral organs and the reproductive system observed.

E. FERTILITY INDICES

In the first mating interval (study week 1), the number and percentage of pregnant females was reduced in the high dose group (refer to Table B.6.4.3.1-3). The effect was explained by acute toxicity of the test item and in line with increased incidences of early resorptions (refer to Table B.6.4.3.1-6), pre- and post-implantation losses (refer to Table B.6.4.3.1-10 and Table B.6.4.3.1-11). Pregnancy status at 200 and 1000 mg/kg bw was not affected by treatment. Within the remaining 9 mating intervals, there were no changes in the number and percentage of pregnant females.

Table B.6.4.3.1-3: Dominant lethal test in Wistar rats ales ([redacted], 1992), number and percentage of pregnant females

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
No. of pregnant females (%)					
Mating study week					
1	29 (97)	28 (93)	29 (97)	23 (77)	10 (100)
2	25 (83)	29 (97)	23 (77)	26 (87)	8 (80)
3	27 (90)	26 (87)	26 (87)	25 (83)	0 (0)
4	28 (93)	29 (97)	26 (87)	26 (87)	2 (22)
5	27 (90)	29 (97)	28 (93)	27 (90)	6 (67)
6	27 (90)	27 (90)	26 (87)	29 (97)	7 (78)
7	28 (93)	23 (77)	29 (97)	25 (83)	7 (78)
8	28 (93)	26 (87)	27 (90)	29 (97)	8 (89)
9	24 (80)	26 (87)	28 (93)	30 (100)	8 (89)
10	25 (83)	59 (97)	28 (93)	29 (97)	9 (100)

PC: positive control EMS: Ethylmethanesulphonate, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

The number of *corpora lutea* per dam was not affected by treatment (refer to Table B.6.4.3.1-4). A decreased number of *corpora lutea* at 1000 mg/kg bw among the females of the first mating interval and a significant increase in the number of *corpora lutea* at 5000 mg/kg bw in the females of the third mating interval were considered incidental.

Table B.6.4.3.1-4: Dominant lethal test in Wistar rats ([redacted], 1992), number of *corpora lutea*

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
No. of *corpora lutea*: Mean per dam ± SD					
Mating study week					

Table B.6.4.3.1-4: Dominant lethal test in Wistar rats ([redacted] 1992), number of *corpora lutea*

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
1	12.4 ± 1.66	12.1 ± 1.27	11.1 ± 2.03* ↓	12.4 ± 3.58	11.0 ± 2.14*
2	11.6 ± 1.98	11.5 ± 1.96	10.4 ± 1.41	12.3 ± 2.26	12.0 ± 6.65
3	10.9 ± 1.99	11.2 ± 2.54	9.88 ± 1.58	12.4 ± 1.44* ↑	0.0 ± 0.0
4	10.3 ± 1.70	11.9 ± 2.01	12.2 ± 2.51	11.7 ± 2.61	12.0 ± 5.08*
5	11.7 ±1.54	10.6 ± 1.68	12.4 ± 2.13	12.1 ± 1.40	12.3 ± 6.74
6	11.4 ± 2.04	11.2 ± 2.27	11.4 ± 1.72	11.7 ± 2.44	10.7 ± 5.32
7	14.6 ± 1.95	12.4 ± 1.78	12.9 ± 2.43	11.3 ± 1.67	12.7 ± 6.23*
8	13.2 ± 2.11	13.8 ± 2.26	11.0 ± 1.34	10.6 ± 2.01	12.3 ± 5.29
9	14.2 ± 2.53	12.0 ± 2.16	10.6 ± 1.67	12.1 ± 1.53	11.6 ± 5.17*
10	12.1 ± 2.15	12.1 ± 1.98	12.8 ± 2.38	13.0 ± 1.77	12.6 ± 4.45

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

Upon treatment with glyphosate, the number of implantations per dam showed fluctuations among all dose groups over the entire study period (refer to Table B.6.4.3.1.-51). The statistically significant decrease at 5000 mg/kg bw in females of the first mating interval was attributed to the acute toxic effects caused by the test item and of toxicological relevance. Further, there was a significant increase in the number of implantations per dam during study week 4 at 200 mg/kg bw, a significant decrease in all test item treated groups in study weeks 7 and 8 and in the mid dose group (1000 mg/kg bw) during study week 9. The statistically significant observations in test item-treated groups during mating intervals 4, 7, 8 and 9 were attributed to the high variability of implantation sites among solvent control animals rather than to treatment with glyphosate and therefore considered to be incidental.

Table B.6.4.3.1.-51: Dominant lethal test in Wistar rats ([redacted] 1992), number of implantation sites per dam

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
No. of implantation sites: Mean per dam ± SD					
Mating study week					
1	11.7 ± 2.39	11.4 ± 1.83	10.8 ± 2.02	8.91 ± 3.60* ↓	9.10 ± 3.17*
2	10.3 ± 2.94	11.2 ± 2.54	9.70 ± 2.10	11.9 ± 2.41	2.63 ± 2.42*
3	10.1 ± 2.16	10.5 ± 2.94	8.92 ± 2.37	11.0 ± 2.21	0.0 ± 0.0
4	9.39 ± 2.10	10.6 ± 2.40* ↑	9.73 ± 3.74	10.2 ± 3.10	2.00 ± 0.97*
5	10.8 ± 1.69	10.5 ± 1.81	11.1 ± 3.11	10.7 ± .034	4.83 ± 3.90*
6	10.4 ± 2.21	10.4 ± 2.62	10.7 ± 2.79	10.1 ± 2.64	7.57 ± 4.62*
7	13.5 ± 2.36	10.1 ± 3.50*↓	11.0 ± 4.06* ↓	9.88 ± 2.65* ↓	11.4 ± 6.22*
8	11.7 ± 3.40	12.0 ± 3.19	10.4 ± 1.42* ↓	9.41 ± 2.29* ↓	10.0 ± 5.12*
9	12.0 ± 3.75	11.0 ± 2.51	9.57 ± 2.10* ↓	11.1 ± 2.83	11.1 ± 5.13
10	10.8 ± 2.66	11.3 ± 2.90	10.9 ± 3.38	12.3 ± 2.75	11.9 ± 4.19

Table B.6.4.3.1.-51: Dominant lethal test in Wistar rats ([redacted] 1992), number of implantation sites per dam

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw]				
	0	**200**	**1000**	**5000**	**100/500[x]**

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

A statistically significant increase in the number of early resorptions was noted during study week 1 (first mating) at 5000 mg/kg bw, during study week 3 (third mating) at ≥ 1000 mg/kg bw and during study week 4 (fourth mating) at 1000 mg/kg bw. The effect was not reproducible in the next mating interval. Due to a lack of a dose-dependency, the observation was not attributed to treatment.

Table B.6.4.3.1-6: Dominant lethal test in Wistar rats ([redacted] 1992), number of total early resorptions per group

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw]				
	0	**200**	**1000**	**5000**	**100/500[x]**
No. of early resorptions: Number of total early resorptions per group[#]					
Mating study week					
1	0.0	0.0	0.0	25.0* ↑	0.0
2	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	10.0	8.0	0.0
4	0.0	0.0	24.0* ↑	0.0	0.0
5	0.0	0.0	0.0	1.0	0.0
6	0.0	0.0	0.0	0.0	0.0
7	0.0	1.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

[#]: reported as mean number of early resorptions per dam in study report

There was a statistically significant increase in the incidence of small moles / fetal resorptions at ≥ 1000 mg/kg bw during weeks 1 and 7, at 1000 mg/kg bw during week 5, at 5000 mg/kg bw during weeks 6 and 8 and in all glyphosate treated groups during week 9 (refer to Table B.6.4.3.1-7). In addition, strong fluctuations in the percentages of fetal resorptions were noted over the entire study period. The observations were not dose-related and inconsistent throughout the study period and therefore not attributed to treatment.

Table B.6.4.3.1-7: Dominant lethal test in Wistar rats ([redacted] 1992), mean number of fetal resorptions per dam and percentage of fetal resorptions per group (small moles)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
Small moles / fetal resorptions: Mean per dam ± SD					
Mating study week					
1	0.38 ± 0.68	0.29 ± 0.53	0.62 ± 0.86* ↑	0.78 ± 1.17* ↑	2.80 ± 2.71*
2	0.92 ± 2.71	0.34 ± 0.67* ↓	0.35 ± 0.57* ↓	0.88 ± 1.63	1.80 ± 1.72
3	0.96 ± 2.59	0.58 ± 1.17* ↓	0.85 ± 1.32	0.72 ± 0.89	0.0 ± 0.0
4	0.71 ± 0.98	0.55 ± 0.91	0.62 ± 0.90	0.81 ± 1.27	2.00 ± 0.97*
5	0.59 ± 0.97	0.69 ± 0.81	0.86 ± 1.48* ↑	0.59 ± 1.05	0.83 ± 1.58
6	0.37 ± 0.56	0.44 ± 0.80	0.46 ± 0.58	1.10 ± 1.47* ↑	1.00 ± 1.25
7	0.43 ± 0.84	0.48 ± 0.49	0.62 ± 0.98* ↑	1.01 ± 1.14* ↑	0.71 ± 1.08
8	0.57 ± 1.26	0.12 ± 0.33* ↓	0.67 ± 0.88	1.00 ± 1.25* ↑	0.50 ± 0.70
9	0.13 ± 0.34	0.81 ± 1.39* ↑	0.32 ± 0.67* ↑	0.87 ± 0.97* ↑	0.25 ± 0.42
10	0.52 ± 0.77	0.21 ± 0.41* ↓	0.07 ± 0.26* ↓	0.45 ± 0.57	0.56 ± 0.85
Percentage of small moles / fetal resorptions					
Mating study week					
1	3.04 ± 5.33	2.48 ± 5.04	5.46 ± 7.54* ↑	10.9 ± 18.2* ↑	33.5 ± 34.7*
2	11.7 ± 29.5	3.05 ± 5.79* ↓	3.22 ± 5.30* ↓	7.87 ± 16.1* ↓	82.3 ± 42.1*
3	10.6 ± 27.3	6.22 ± 12.8* ↓	10.5 ± 14.4	8.32 ± 14.4* ↓	0.0 ± 0.0
4	7.85 ± 11.3	5.14 ± 8.59* ↓	10.1 ± 21.0* ↑	6.55 ± 8.97* ↓	100 ± 42.2*
5	5.21 ± 8.52	6.34 ± 7.25* ↑	9.11 ± 18.4* ↑	7.44 ± 16.5* ↑	16.7 ± 31.6*
6	3.17 ± 4.79	3.88 ± 6.76	5.45 ± 10.3* ↑	11.0 ± 16.7* ↑	12.2 ± 13.5*
7	3.15 ± 6.18	10.4 ± 22.6* ↑	8.30 ± 19.8* ↑	11.1 ± 13.6* ↑	10.0 ± 16.4*
8	6.05 ± 14.0	0.87 ± 2.45* ↓	6.15 ± 7.97	13.0 ± 21.0* ↑	4.3 ± 5.98*
9	0.99 ± 2.67	8.87 ± 18.2*	3.58 ± 8.03*	7.72 ± 8.74*	2.34 ± 4.02*
10	4.41 ± 6.15	2.39 ± 5.52*	0.60 ± 2.21*	5.11 ± 9.88*	4.26 ± 6.77

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

A statistically significant increase in the mean number of embryonic resorptions per dam was observed in the low and mid dose group only for mating intervals 3, 4 and 10 (refer to Table B.6.4.3.1-8 Table below). In addition, the percentage of embryonic resorptions was statistically significantly increased at 5000 mg/kg bw after the first mating, at 200 mg/kg bw after the 2nd, 3rd and 8th mating and at 200 and 1000 mg/kg bw during weeks 4 and 10. Without any dose-response relationship, the observations were considered to be incidental and not related to treatment.

Table B.6.4.3.1-8: Dominant lethal test in Wistar rats ([redacted], 1992) mean number of embryonic resorptions per dam and percentage of embryonic resorptions per group (large moles)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
Large moles / embryonic resorptions: Mean per dam ± SD					
Mating study week					
1	0.31 ± 0.60	0.36 ± 0.56	0.0 ± 0.0	0.17 ± 0.49	0.60 ± 1.50
2	0.52 ± 0.82	0.52 ± 0.74	0.09 ± 0.42* ↓	0.12 ± 0.33* ↓	0.50 ± 0.84
3	0.37 ± 0.63	0.62 ± 0.98	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	0.11 ± 0.31	0.38 ± 0.68* ↑	0.38 ± 0.98* ↑	0.15 ± 0.54	0.0 ± 0.0
5	0.44 ± 0.97	0.28 ± 0.59* ↓	0.25 ± 0.52* ↓	0.30 ± 1.35	1.17 ± 1.25
6	0.44 ± 0.97	0.19 ± 0.40	0.35 ± 0.80	0.0 ± 0.0	0.43 ± 0.67
7	0.75 ± 0.75	0.74 ± 1.18	0.52 ± 0.78	0.0 ± 0.0	1.14 ± 1.23
8	0.79 ± 0.88	0.62 ± 0.80* ↓	0.26 ± 0.53* ↓	0.03 ± 0.19* ↓	1.00 ± 1.03
9	1.46 ± 1.56	0.69 ± 0.97	0.29 ± 0.53* ↓	0.0 ± 0.0* ↓	0.50 ± 0.70
10	0.16 ± 0.47	0.38 ± 0.73* ↑	0.36 ± 0.62* ↑	0.17 ± 0.38	0.44 ± 0.70
Percentage of large moles / embryonic resorptions					
Mating study week					
1	2.69 ± 5.64	3.31 ± 5.36	0.0 ± 0.0* ↓	3.35 ± 10.9* ↑	10.9 ± 29.8*
2	5.50 ± 9.47	7.09 ± 18.8* ↑	0.65 ± 3.13* ↓	1.08 ± 3.25* ↓	14.6 ± 25.0
3	3.91 ± 6.51	5.54 ± 9.03* ↑	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4	0.91 ± 2.70	3.63 ± 6.33* ↑	3.98 ± 11.1* ↑	1.38 ± 5.02	0.0 ± 0.0*
5	3.84 ± 8.04	2.58 ± 5.43* ↓	2.10 ± 4.46* ↓	2.52 ± 11.3* ↓	32.0 ± 33.3*
6	4.25 ± 9.45	2.13 ± 5.02* ↓	4.84 ± 15.1	0.0 ± 0.0* ↓	3.86 ± 5.93
7	5.90 ± 6.22	6.33 ± 9.44	4.61 ± 6.75* ↓	0.0 ± 0.0	11.6 ± 11.7*
8	7.18 ± 9.69	9.48 ± 20.7* ↑	2.57 ± 5.40* ↓	0.45 ± 2.41* ↓	11.4 ± 13.4*
9	12.1 ± 12.5	7.08 ± 9.45* ↓	3.01 ± 5.53* ↓	0.0 ± 0.0* ↓	3.73 ± 5.23*
10	1.36 ± 3.88	3.78 ± 7.58* ↑	3.01 ± 5.17* ↑	1.25 ± 2.80	3.93 ± 6.64*

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

Increases and decreases in the incidence and percentage of live implants were observed in test item-treated and solvent control-treated animals and occurred without any dose-relation and without consistency over the 10 mating intervals. Thus, the changes were as well considered unrelated to treatment (refer to Table B.6.4.3.1-9).

Table B.6.4.3.1-9: Dominant lethal test in Wistar rats ([redacted] 1992), mean number of live implants per dam and per centage of live implants per group

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw]				
	0	**200**	**1000**	**5000**	**100/500[x]**
Live implants: Mean per dam ± SD					
Mating study week					
1	11.0 ± 2.38	10.8 ± 1.97	10.2 ± 1.98	6.87 ± 5.08* ↓	6.50 ± 4.79*
2	8.88 ± 3.78	10.3 ± 2.67* ↑	9.26 ± 1.89	10.9 ± 3.01* ↑	0.25 ± 0.63*
3	8.81 ± 3.48	9.35 ± 3.11	7.69 ± 2.96	9.92 ± 3.23	0.0 ± 0.0
4	8.57 ± 2.15	9.69 ± 2.52* ↑	7.81 ± 5.01	9.23 ± 2.42	0.0 ± 0.0*
5	9.74 ± 1.83	9.52 ± 1.60	9.96 ± 3.58	9.78 ± 3.61	2.83 ± 3.20*
6	9.63 ± 2.17	9.78 ± 2.47	9.92 ± 2.99	9.03 ± 2.86	6.14 ± 3.68*
7	12.3 ± 2.77	8.83 ± 3.52	9.86 ± 4.02	8.84 ± 2.72	9.57 ± 5.72*
8	10.4 ± 3.87	11.3 ± 3.53	9.48 ± 1.63	8.38 ± 2.78* ↓	8.50 ± 4.54*
9	10.4 ± 3.59	9.46 ± 3.42	8.96 ± 2.24	10.2 ± 2.85	10.4 ± 4.72
10	10.1 ± 2.49	10.7 ± 2.93	10.5 ± 3.32	11.7 ± 2.77	10.9 ± 3.91
Percentage of live implants					
Mating study week					
1	94.3 ± 7.27	94.3 ± 6.69	94.6 ± 7.53	64.9 ± 43.3* ↓	55.6 ± 39.0*
2	82.8 ± 28.7	89.8 ± 18.8* ↑	96.1 ± 6.66* ↑	91.2 ± 16.0* ↑	3.13 ± 7.91*
3	85.4 ± 26.8	88.2 ± 15.1	85.7 ± 22.6	87.7 ± 23.2	0.0 ± 0.0
4	91.4 ± 10.8	91.2 ± 9.58	71.0 ± 38.0* ↓	92.1 ± 9.32	0.0 ± 0.0*
5	91.0 ± 12.4	91.1 ± 8.32	88.8 ± 18.2	89.4 ± 19.0	51.3 ± 41.9*
6	92.7 ± 10.7	94.1 ± 7.47	89.7 ± 16.9* ↓	89.0 ± 16.7* ↓	84.0 ± 42.2*
7	90.9 ± 10.2	82.90 ± 22.0* ↓	87.1 ± 19.1* ↓	89.0 ± 13.5	78.3 ± 43.7*
8	87.7 ± 16.4	89.7 ± 20.4	91.3 ± 10.3* ↑	86.6 ± 20.8	84.4 ± 37.6*
9	86.9 ± 12.6	84.1 ± 23.4* ↓	93.5 ± 8.92*	92.3 ± 8.74* ↑	94.0 ± 40.1*
10	94.2 ± 6.86	93.9 ± 8.73	96.4 ± 6.64	93.7 ± 9.98	91.9 ± 30.7*

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

Statistically significant increases in the percentages of pre-implantation losses were noted at 5000 mg/kg bw for the first mating interval, for all or most treatment groups during weeks 3, 4, 5 and 7 and statistically significantly decreased in all or in the majority of glyphosate treated groups during weeks 2, 8, 9 and 10. There was no dose-response relationship evident at any mating interval. Thus, the effects on the percentage of pre-implantation losses were not considered to be related to treatment with glyphosate.

Table B.6.4.3.1-10: Dominant lethal test in Wistar rats ([redacted] 1992), percentage of pre-implantation losses

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
Percentage of pre-implantation losses (%): Mean per dam ± SD					
Mating study week					
1	6.21 ± 13.5	5.54 ± 11.7	2.37 ± 5.34* ↓	24.2 ± 30.2* ↑	12.3 ± 19.5*
2	10.6 ± 20.1	3.69 ± 14.1* ↓	7.75 ± 12.0* ↓	3.98 ± 7.31* ↓	73.0 ± 38.8*
3	6.60 ± 9.65	6.81 ± 13.0	10.1 ± 17.8* ↑	10.9 ± 16.5* ↑	0.0 ± 0.0
4	8.36 ± 14.6	9.97 ± 14.6* ↑	21.3 ± 24.7* ↑	12.8 ± 19.9* ↑	82.5 ± 35.1*
5	7.57 ± 10.7	1.49 ± 3.82*↓	11.6 ± 18.0* ↑	12.1 ± 21.9* ↑	60.8 ± 40.3*
6	8.56 ± 10.5	6.59 ± 14.6* ↓	7.56 ± 17.8	12.5 ± 18.5* ↑	30.0 ± 29.5*
7	7.79 ± 11.1	18.2 ± 26.4* ↑	15.0 ± 25.1* ↑	13.1 ± 19.1* ↑	10.7 ± 21.1*
8	12.1 ± 18.9	13.8 ± 19.8* ↑	5.62 ± 6.59* ↓	9.72 ± 18.9* ↓	19.1 ± 22.4*
9	16.8 ± 21.4	8.12 ± 13.9* ↓	9.47 ± 14.6* ↓	9.20 ± 18.7* ↓	4.63 ± 8.77*
10	11.3 ± 14.3	7.20 ± 16.4* ↓	14.8 ± 24.5* ↑	6.17 ± 17.5* ↓	4.92 ± 6.92*

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

The percentage of post-implantation loss, corresponding to the dominant lethal factor, was statistically significantly increased in the high dose group of the first mating interval, which was attributed to acute toxicity in the animals. The dominant lethal factor was further statistically significantly increased in the mid and high dose group after the 6th mating and for all glyphosate treated groups after the 7th mating. For the females of the remaining mating intervals, the dominant lethal factor was either not affected or lower than in solvent control animals. None of the observations was dose-related or consistent over the ten mating periods.

Table B.6.4.3.1-11: Dominant lethal test in Wistar rats ([redacted] 1992), percentage of post-implantation losses, corresponding to dominant lethal factors

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
Percentage of post-implantation losses[#] (%)					
Mating study week					
1	5.71 ± 7.28	5.76 ± 6.74	5.46 ± 7.54	35.1 ± 43.3* ↑	44.4 ± 39.0*
2	17.2 ± 28.8	10.2 ± 18.8*	3.89 ± 6.67* ↓	8.93 ± 16.0* ↓	96.9 ± 41.6*
3	14.5 ± 26.8	11.8 ± 15.1* ↓	14.3 ± 22.6	12.3 ± 23.2	0.0 ± 0.0
4	8.76 ± 10.9	8.79 ± 9.58	29.0 ± 38.0* ↑	7.93 ± 9.32	100 ± 42.2*
5	9.07 ± 12.4	8.88 ± 8.34	11.2 ± 18.1* ↑	10.6 ± 19.0	48.7 ± 41.0*
6	7.43 ± 10.8	6.03 ± 7.55* ↓	10.3 ± 16.9* ↑	11.0 ± 16.7* ↑	16.0 ± 14.0*

Table B.6.4.3.1-11: Dominant lethal test in Wistar rats ([redacted], 1992), percentage of post-implantation losses, corresponding to dominant lethal factors

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	1000	5000	100/500[x]
7	9.10 ± 10.2	17.2 ± 22.0* ↑	12.9 ± 19.1* ↑	11.1 ± 13.6* ↑	21.7 ± 24.2*
8	13.2 ± 15.9	10.3 ± 20.5* ↓	8.71 ± 10.3* ↓	13.4 ± 20.8	15.7 ± 13.8*
9	13.1 ± 12.6	16.0 ± 23.4* ↑	6.59 ± 8.95* ↓	7.72 ± 8.74* ↓	6.01 ± 6.58*
10	5.77 ± 6.86	6.17 ± 8.74	3.59 ± 6.62	6.34 ± 9.98	8.22 ± 10.3*

PC: positive control EMS: Ethylmethanesulphonate, positive control, [x]combined data for both treatment regimens (100 mg/kg bw/day for 5 days in distilled water or 500 mg/kg bw as single dose in distilled water)

[#]: Corresponds to the dominant lethal factor

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls, ↓ decrease, ↑ increase

Treatment with the positive control ethylmethane sulphonate induced statistically significant dominant lethal effects and demonstrated the validity of the test. There was a statistically significant decrease in the incidence of pregnancy to 0 % observed in the third mating interval and a gradual recovery back to normal by 9 to 10 weeks (refer to Table B.6.4.3.1-3B.6.4.3.1-3). In addition, there was an increased incidence of small moles / fetal resorptions during the first week (mating period 1), which gradually recovered to normal by week 9 (mating period 9, refer to Table B.6.4.3.1-7).

III. CONCLUSION

Under the conditions of the test, glyphosate did not induce dominant lethal effects in Wistar rats up to a dose level of 5000 mg/kg bw. Treatment of males with 5000 mg/kg bw was associated with clinical signs of toxicity and reduced body weight in males, as well as with acute toxicity after the first mating in females. Toxicity in female rats became evident as a reduction in the incidence of pregnancy and an increase in the incidences of early resorption and pre- and post-implantation losses. Based on the experimental findings, glyphosate was considered non-genotoxic to germ cells *in vivo*.

Assessment and conclusion by applicant:

Negative for genotoxicity to germ cells *in vivo*. The test item did not induce any dominant lethal effects.

At 5000 mg/kg bw, treatment with glyphosate caused acute toxic effects in females of the first mating group, which affected the fertility indices pregnancy, early resorption, pre- and post-implantation losses.

The study was performed under GLP and in accordance with OECD guideline 478 (1984). When compared to the current OECD guideline (2016), a number of deviations became evident. The spacing factor between dose levels was too high (5 instead of 2 – 4) and the number of implantations per group for each mating period was below the recommended number of 400 implants per group as required according to OECD 478 (2016). Fetal body weights were not recorded and no information on historical control data was provided. Further, there was no raw data on individual animals treated with the positive control provided. The mean pre- and post-implantation losses per dam were not calculated, but percentages of pre-implantation losses, as well as percentages of post-implantation losses (corresponding to the dominant lethal factor) were reported. Further deviations were considered to be of minor degree. Due to the large number of deviations to the current OECD guideline, the study was considered to provide supporting information.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for genotoxicity to germ cells *in vivo*. The test item did not induce any dominant lethal effects under the conditions

of this test. None of the observations was dose-related or consistent over the ten mating periods. The study is considered supportive due to the noted deviations.

B.6.4.3.1. *In vivo study in germ cells – Dominant lethal assay, study 2*

Data point	CA 5.4.3/004
Report author	[redacted]
Report year	1982
Report title	Mutagenic testing of glyphosate in rat by dominant lethal test
Report No	Not reported
Document No	Not reported
Guidelines followed in study	No guideline followed, study was conducted similarly to OECD 478 (2016)
Deviations from current test guideline OECD 478 (2016)	Although OECD 478 does not state the recommended age of the animals, rather old animals (12 months at study initiation) were used in this study. When compared to OECD 478 (2016), clinical signs of toxicity and mortality were not reported and no body weights were recorded. The selected dose levels were comparably low, but a scientific justification for the selection of dose levels was not given. For some mating intervals, the number of implantations per group for each mating period was below the recommended number of 400 implants per group. Therefore, the number of animals mated was presumably not sufficient. Foetal body weights were not recorded and no historical control data were provided. Necropsy of sires was not performed and resorptions, termed as "dead implants" in study report were not distinguished as early or late resorptions. In addition, pregnant females were sacrificed on gestation Day 18, whereas OECD 478 (2016) recommends to sacrifice the females between gestation Days 14 – 15 for rats. Raw data on individual animals were not provided, therefore standard deviations were not calculated. In addition, analytical determinations on the test items stability, homogeneity or concentration in the diet were not performed. Acceptance and evaluation criteria were not specified in the study report.
Previous evaluation	Not accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, not conducted under GLP /Officially recognised testing facilities. When the study was performed, GLP was not compulsory.
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 3a **Conclusion AGG:** The study is considered supportive due to the noted deviations.

The mutagenic potential of glyphosate active principle (batch: 00260481, purity: not reported) was investigated in a dominant lethal study in CFY rats.

The test item was incorporated into the ground diet and administered via the feed to male animals for a period of 8 weeks. Dose levels of 10, 30 and 100 mg glyphosate/kg food were applied, corresponding to a mean actual achieved test substance intake of 6.8, 20.5 and 70.4 mg/kg bw/day. A group of control males received the normal diet without test item and a group of positive control animals was administered 5 mg cyclophosphamide/kg food, corresponding to a mean actual achieved intake of 3.7 mg/kg bw/day.

After dosing, each male was paired with untreated females (mating ratio 1 : 2) for one week. After separation, the males were paired with two new females for a second week. Females were continually replaced after one week until 4 mating intervals were completed.

Information on clinical signs of toxicity, mortality or individual and group mean body weights were not included in the study. Food consumption and mean achieved compound intake was recorded on a weekly base. Analytical determinations on stability, homogeneity and concentration of the test item in the diet were not conducted.

Females were sacrificed on Day 18 of gestation. The uterus and ovaries were dissected and investigated for the number of *corpora lutea*, the number of implantations, the number of dead implants (resorptions) and the number of viable fetuses.

Investigations of the female uterine contents revealed no treatment-related findings. The number of pregnant females, the number of *corpora lutea* and implantation sites and the number of resorptions were not affected for any mating interval upon treatment with glyphosate. The number of viable fetuses was comparable for females mated with glyphosate-treated and solvent control-treated males. In addition, the number of females having one or more dead fetus did not increase. The percentage of pre-implantation loss was considered not to be affected by treatment. Fluctuations were noted in females of all dose groups, but most groups showed a decrease in pre-implantation losses. However, the findings were not consistent during the 4-week mating period and a dose-response relationship was not observed. The percentage of post-implantation loss, corresponding to the dominant lethal factor, was comparable for all glyphosate and control groups.

Treatment with cyclophosphamide resulted in a strong increase in the number of dead implants / resorptions in the females of all mating intervals and a reduction in the number of viable fetuses, especially in the females of the first mating interval. Cyclophosphamide proved to be highly mutagenic and induced clear dominant lethal effects, demonstrating the sensitivity and validity of the test.

Based on the experimental findings, glyphosate active principle has no mutagenic potential in germ cells *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material: N-phosphonomethyl-glycine

Identification:	Glyphosate active principle
Description:	Not specified
Lot/Batch #:	00260481
Purity:	Not specified
Stability of test compound:	The stability of the test item at storage conditions or in vehicle (feed) was not specified.
Solvent (vehicle) used:	The test item was incorporated into the diet (LATI laboratory rodent food)

2. Control materials

Solvent (vehicle) control:	LATI laboratory rodent food
Positive control:	Cyclophosphamide, 5 mg/kg food (nominal concentration), corresponding to 3.7 mg/kg bw/day (actual dose received)

3. Test animals:

Species:	Rat
Strain:	CFY
Sex:	Males and females
Source:	[redacted]
Age at study initiation:	12 months
Weight at study initiation:	Not specified
Acclimation period:	Not specified
Diet/Food:	Standard LATI rat and mouse food (Lati, Gödöllö, Hungary)
Water:	Not specified

Housing:	In groups of 5/sex in shoebox type plastic cages with wood shaving bedding. During mating: Each male was cohabitated with 2 females.

4. Environmental conditions:

Environmental conditions were not specified.

5. Test concentrations and treatment groups:

Dose levels:	10, 30 and 100 mg/kg food (nominal dose), corresponding to 6.8, 20.5 and 70.4 mg/kg bw/day (actual dose, calculated based on average food intake)
Treatment duration:	8 weeks
Number of animals:	The total number of animals used was 100 males and 900 females. The number of animals used per mating interval was not explicitly specified in the study report. As the animals were divided into 5 groups, it can be assumed that 20 males per group were used. There is a discrepancy in the mentioned number of 900 females used and the information that each male was mated during the 4 week mating period with 2 females per week, leading to a total number of 800 females used in the study.
Route of administration:	Oral, feed

B: STUDY DESIGN AND METHODS

1. Finalisation date: 1982

2. Animal assignment and treatment:

The test item was incorporated into the ground diet and administered via the feed to male animals for a period of 8 weeks. Dose levels of 10, 30 and 100 mg/kg food were applied, corresponding to an actual mean daily intake of 6.8, 20.5 and 70.4 mg/kg bw/day, as calculated based on the average weekly food intake per group. A group of control males received the normal diet without test item and a group of positive control animals was administered 5 mg/kg food cyclophosphamide, corresponding to 3.7 mg/kg bw/day (actual dose received).
After dosing, each male was paired during 4 weeks with two females per week (mating ratio 1 : 2).

3. Observations

Information on observations regarding mortality or clinical signs of toxicity were not included in the study report.

4. Body weight

Information on individual or group mean body weights of animals in the respective groups were not included in the study report.

5. Food consumption and compound intake

Food consumption and test substance intake were recorded for each dose group in mg/kg bw/week.

6. Sacrifice

Female rats were sacrificed on the 18th day of gestation. The uteri and ovaries of the animals were dissected and investigated for the following parameters:

- Number of *corpora lutea*
- Number of implantations
- Number of resorptions
- Number of viable and nonviable fetuses

Furthermore, the percentage of pre- and post-implantation loss was determined as follows:

$$\text{Pre} - \text{implantation loss } [\%] = \frac{\text{Total number of corpora lutea} - \text{Number of implantations}}{\text{Total number of corpora lutea}} \times 100$$

$$\text{Post} - \text{implantation loss } [\%] = \frac{\text{Number of implantations} - \text{Number of live fetuses} *}{\text{Total number of implantations}} \times 100$$

* fetuses (gestation day 18) were termed embryos in study report

The percentage of post-implantation losses corresponds to the dominant lethal factor.

Macroscopical examination of male rats was not included in the study report.

7. Statistics:

Statistical analysis was not described in the study report.

8. Acceptance criteria:

Acceptance criteria were not specified in the study report.

9. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations on the stability, concentration and homogeneity of the test substance in the diet were not performed in the study.

B. OBSERVATIONS

Systemic toxicity:

Mortality:

Information on mortality was not provided in the study report.

Clinical signs of toxicity:

Clinical signs of toxicity were not reported.

C. FOOD CONSUMPTION AND COMPOUND INTAKE

There were no treatment-related effects on food consumption of males during the 8-week treatment period. The average intake of test substance per group was 47.3 ± 2.9, 143.3 ± 11.9 and 492.9 ± 35.4 mg/kg bw/week, calculated based on the average food intake (g/kg bw/week) over all treatment weeks. Based on this, the corresponding mean daily doses of 6.8, 20.5 and 70.4 mg/kg bw/day were calculated.

D. BODY WEIGHT AND BODY WEIGHT DEVELOPMENT OF SIRES

Body weights were not recorded during the course of the study.

E. NECROPSY OF SIRES

Information on macroscopical or microscopical analysis of sacrificed males was not included.

F. FERTILITY INDICES

Treatment with glyphosate did not affect the number of mated males and females (refer to Table B.6.4.3.4-1 and Table B.6.4.3.4-2). The number of pregnant females was comparable for all groups.

Table B.6.4.3.4-1: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of males mated

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
No. of males mated					
Mating study week					
1	15	20	19	18	20
2	18	19	19	19	20
3	18	17	19	19	20
4	17	17	18	19	18

PC: positive control Cyclophosphamide

Table B.6.4.3.4-2: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of pregnant females

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
No. of pregnant females					
Mating study week					
1	23	33	29	29	32
2	30	31	30	36	34
3	29	26	28	29	32
4	28	25	32	33	31

PC: positive control Cyclophosphamide

In addition, the number of *corpora lutea* and the number of implantations (refer to Table B.6.4.3.4-3 and Table B.6.4.3.4-4) were not affected by treatment.

Table B.6.4.3.4-3: Mutagenic testing of glyphosate in rat by dominant lethal test [redacted] 1982), number of *corpora lutea*

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
No. of corpora lutea: Mean per dam					
Mating study week					
1	16.7	16.2	17.0	17.2	16.6
2	16.6	15.3	16.5	16.0	17.1
3	16.2	16.3	16.2	17.2	17.5

Table B.6.4.3.4-3: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of *corpora lutea*

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
4	15.9	16.5	16.8	15.8	16.1

PC: positive control Cyclophosphamide

Table B.6.4.3.4-4: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of implantation sites

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
No. of implantation sites: Mean per dam					
Mating study week					
1	13.6	14.8	15.0	13.7	13.6
2	13.5	14.2	14.3	13.6	14.4
3	13.5	13.7	14.5	13.5	14.1
4	13.6	15.0	14.7	13.9	14.7

PC: positive control Cyclophosphamide

The amount of resorptions (termed “dead implants” in study report) was comparable to those of control animals for all mating intervals (refer to Table B.6.4.3.4-5). The number of females having one or more dead fetus of early death did not increase either.

Table B.6.4.3.4-5: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of dead implants / resorptions

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
Number of dead implants / resorptions: Mean per dam					
Mating study week					
1	1.7	1.1	1.3	1.3	7.7
2	1.3	1.2	1.2	1.4	6.2
3	1.7	1.5	1.1	1.5	4.2
4	1.7	2.2	1.1	1.5	3.5

PC: positive control Cyclophosphamide

The number of viable fetuses (termed “live implants” or “live embryos” in study report) in glyphosate-treated animals was comparable to those of control animals.

Table B.6.4.3.4-6: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), number of live implants / viable foetuses

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
Number of live implants / viable fetuses: Mean per dam					
Mating study week					
1	11.8	13.7	13.7	12.4	5.8
2	12.2	13.1	13.1	12.3	8.2
3	11.9	12.2	13.4	12.0	9.9
4	11.9	12.8	13.6	12.5	11.2

PC: positive control Cyclophosphamide

Further, the percentage of pre -implantation losses (refer to Table B.6.4.3.4-7) was not considered to be affected upon treatment with glyphosate. Fluctuations were noted in females of all dose groups, but most groups showed a decrease in pre-implantation losses. However, the findings were not consistent during the 4-weeks mating period and a dose-response relationship was not observed.

Table B.6.4.3.4-7: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), percentage of pre-implantation losses

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
Percentage of pre-implantation losses (%)					
Mating study week					
1	19.0	9.0 ↓	11.6 ↓	20.2	18.1
2	18.5	7.6 ↓	13.3 ↓	14.9 ↓	16.0
3	16.8	16.3	10.6 ↓	21.8 ↑	19.5
4	14.4	9.2 ↓	12.5 ↓	11.7 ↓	8.4

PC: positive control Cyclophosphamide

The percentage of post-implantation losses, corresponding to the dominant lethal factor, was not affected by treatment (refer to Table B.6.4.3.4-8).

Treatment with the positive control cyclophosphamide markedly increased the number of dead implants / resorptions in the females of all mating intervals (refer to Table B.6.4.3.4-5B.6.4.3.4-5 and markedly reduced the number of viable fetuses, especially in the females of the first mating interval (refer to Table B.6.4.3.4-6). Treatment with cyclophosphamide induced clear dominant lethal effects, demonstrating the sensitivity and validity of the test.

Table B.6.4.3.4-8: Mutagenic testing of glyphosate in rat by dominant lethal test ([redacted] 1982), percentage of post-implantation losses, corresponding to dominant lethal factors

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw/day]				
	0	**6.8**	**20.5**	**70.4**	**3.7**
Percentage of post-implantation losses[#] (%):					
Mating study week					
1	12.8	7.2	8.7	9.8	56.9
2	9.6	7.7	8.4	10.0	42.9
3	12.0	10.4	7.2	10.7	29.9
4	12.6	14.4	7.6	10.0	23.9

PC: positive control Cyclophosphamide

[#]: Corresponds to the dominant lethal factor

III. CONCLUSION

Based on the experimental findings and under the conditions of the test, glyphosate active principle did not cause dominant lethal effects in CFY rats after 8-week dietary administration. Therefore, glyphosate active principle is considered non-genotoxic to germ cells *in vivo*.

Assessment and conclusion by applicant:

Negative for mutagenic effects in the dominant lethal assay in rats.

The study is not compliant with GLP but was performed according to the main criteria of OECD guideline 478 (2016). The study is considered supplementary due to serious reporting deficiencies, e.g. the purity of the test compound was not given and it is not unequivocally clear how many males and females were allocated to the individual test groups. Further methodology deficiencies are the number of implants (< 400/group) indicating the number of animals mated was not sufficient. In addition, the dose levels appear to be too low for definitive assessment. It is known from other studies that much higher doses can be applied. However, it is the only dominant lethal test with repeated dietary administration, and, therefore, the study may provide some additional information.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for for mutagenic effects in the dominant lethal assay in rats under the conditions of this test. Although no statistical analysis was performed none of the observations was dose-related or consistent over the mating periods.
The study is considered supportive with restrictions due to the noted deviations.

B.6.4.3.2. *In vivo study in germ cells – Dominant lethal assay, study 3*

Data point	CA 5.4.3/005
Report author	[redacted]
Report year	1980
Report title	Dominant lethal mutagenicity assay with technical Glyphosate in mice
Report No	401-064
Document No	M-643921-01-1
Guidelines followed in study	No guideline followed, study was conducted similarly to OECD 478 (2016)
Deviations from current test guideline	The number of implantations per group for each mating period was below the recommended number of 400 implants per group. It can be assumed that the number

OECD 478 (2016)	of males and females used for mating was too low. Fetal body weights were not recorded. No historical control data were provided. No dominant lethal frequency was calculated in the study report. Acceptance and evaluation criteria were not specified. Statistical analysis did not include the assessment of under- and overdispersion.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, not conducted under GLP /Officially recognised testing facilities. When the study was performed, GLP was not compulsory.
Acceptability/Reliability	**Conclusion GRG:** Supportive, Category 2a **Conclusion AGG:** The study is considered supportive due to the noted deviations.

The mutagenic potential of glyphosate (batch: XHJ-64, purity: 98.7 %) was investigated in a dominant lethal study in CD-1 mice. The test item was dissolved in aqueous 0.5 % Methocel® and administered by oral gavage to groups of 10 males at dose levels of 200, 800 and 2000 mg/kg bw (single dose) at a constant dosage volume of 10 mL/kg bw. A similar constituted group of males received the solvent control (vehicle) or the positive control Cytoxan®. The positive control substance was administered via intraperitoneal injection as single dose of 240 mg/kg bw at a constant dosage volume of 12 mL/kg bw.

Immediately after dosing, each male was paired with untreated virgin females (mating ratio 1:2) for 7 days. After separation, each male was then paired with two new females for a second week. Females continued to be replaced in this manner for eight weeks so that each male was mated with a total of 16 females. All animals were observed twice daily for mortality and overt changes in appearance and behaviour. Body weights were recorded for males prior to treatment and weekly thereafter. Females were sacrificed 13 days after mid-week of their caging and presumptive mating. The uterus and ovaries were dissected and investigated for the number of *corpora lutea*, the number of implantations, the number of early and late resorptions and the number and localisation of viable and non-viable fetuses.

Four unscheduled deaths were observed during the course of the study. One female mated with a male of the 200 mg/kg bw group died in study week 3, one male of the 800 mg/kg bw group died in study week 5, one male of the 2000 mg/kg bw group died in week 6 and one female mated with a male of the 2000 mg/kg bw dose group died in study week 2. Necropsy of the decedent animals revealed no treatment-related findings, therefore the observations were not attributed to treatment but considered to be incidental. There were no treatment-related clinical signs of toxicity and no differences in body weight development in males over the entire study period.

Investigations of the female uterine contents revealed no treatment-related findings. The number of pregnant females, the number of *corpora lutea* and implantation sites and the number of early and late resorptions were not affected for any mating interval upon treatment with glyphosate. There was a slight but statistically significant decrease in the number of viable fetuses in females of the 800 mg/kg bw group mated during study week 1 and in females of the 2000 mg/kg bw group mated during study week 3. As no increase in early fetal deaths were observed in these groups, the findings were not attributed to glyphosate treatment and considered to be incidental. Pre- and post-implantation losses and calculated dominant lethal factors were comparable for treated and control groups.

Treatment with the positive control Cytoxan® induced a statistically significant decrease in the number of viable fetuses and an increase in the proportion of early fetal deaths, indicated by a statistically significant increase in the mean number of early resorptions and post-implantation loss observed during the first 3 weeks of mating. These results indicated a dominant lethal effect and an effect at the postmeiotic stage of spermatogenesis, thereby demonstrating the validity of the test system.

Under the conditions of the test, there was no mutagenic potential identified for glyphosate in the dominant lethal assay and is therefore considered non-genotoxic to germ cells *in vivo*.

I. MATERIALS AND METHODS

A: MATERIALS

1. Test material:

Identification:	Technical Glyphosate
Description:	White powder
Lot/Batch #:	XHJ-64
Purity:	98.7 %
Stability of test compound:	The stability of the test item at storage conditions or in vehicle was not specified.
Solvent (vehicle) used:	Aqueous 0.5 % Methocel®

2. Control materials

Solvent (vehicle) control:	Aqueous 0.5 % Methocel®
Positive control:	Cytoxan®, 240 mg/kg bw dissolved in sterile water

3. Test animals:

Species:	Mouse
Strain:	CD-1
Sex:	Males and females
Source:	[redacted]
Age at study initiation:	70 - 110 days
Weight at study initiation:	36 – 45 g (males) (weight of females not specified)
Acclimation period:	At least 10 days
Diet/Food:	Purina® Certified Rodent Chow® 5002, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually (except during mating) in suspended wire mesh cages. During mating: Each male was cohabitated with 2 females.

4. Environmental conditions:

The animals were maintained in a temperature-, humidity- and light controlled room

5. Test concentrations and treatment groups:

Dose levels:	200, 800 and 2000 mg/kg bw
Dose volume:	10 mL/kg bw
Number of animals:	10 males and 160 females per group (2 females per mating interval)
Route of administration:	Oral gavage

B: STUDY DESIGN AND METHODS

1. Dates of experimental work: 04 Jun – 09 Aug 1979
Finalisation date: 16 Apr 1980

2. Animal assignment and treatment:

Groups of 10 males per dose received a single administration of glyphosate by oral gavage at dose levels of 200, 800 and 2000 mg/kg bw at a constant dosage volume of 10 mL/kg bw. Similar constituted groups received the solvent (0.5 % aqueous Methocel®). The positive control Cytoxan® was administered via intraperitoneal injection as single dose of 240 mg/kg bw at a constant dosage volume of 12 mL/kg bw.

Immediately after dosing, each male was paired with untreated virgin females (mating ratio 1: 2) for 7 days. After separation, each male was then paired with two new females for a second week. Females continued to be replaced in this manner for eight weeks so that each male was mated with a total of 16 females.

3. Observations

All animals were observed twice daily for mortality and overt changes in appearance and behaviour. All males were given a detailed observation to determine if any clinical signs of toxicity were present.

4. Body weight

The body weight of male animals was recorded prior to treatment and weekly thereafter for 9 weeks.

5. Sacrifice

All mice not surviving to the scheduled sacrifice were necropsied. Males were sacrificed on the last day of mating.

Females were sacrificed by carbon dioxide inhalation thirteen days after mid-week of their caging and presumptive mating. The uterus and ovaries were removed by an abdominal incision and investigated for the following parameters:

- Number of *corpora lutea* per dam
- Number of implantations
- Number of early and late resorptions
- Number and location of viable and nonviable fetuses

The thoracic and abdominal cavities and organs of the dams were examined for grossly evident morphological changes.

Furthermore, the number of post-implantation losses was determined. The number of pre-implantation losses was determined based on the raw data provided in the study report.

Mutagenic effects in the dominant lethal assay were assessed by comparing the number of early fetal deaths in the treated groups versus the control groups. For all groups, the dominant lethal factor was calculated as follows:

$$\text{Dominant lethal factor } [\%] = \frac{\text{Number of post} - \text{implantation losses}}{\text{Total number of implantations}} \times 100$$

6. Statistics:

All statistical analysis compared the treatment groups (test article and positive control) with the vehicle control group.
Fetal deaths per dam and post-implantation losses were compared by the Mann-Whitney U-test. The number of dams with fetal deaths was compared using the chi-square test with Yates' correction for 2 x 2 contingency tables and/or Fisher's exact probability test. The mean number of live fetuses and corpora lutea were compared by analysis of variance (one-way classification) Bartlett's test for homogeneity of variances and the appropriate t-test (for equal or unequal variances) using Dunnett's multiple comparison tables to judge significance of differences.

7. Acceptance criteria:

Acceptance criteria were not specified in the study report.

8. Evaluation criteria:

Evaluation criteria were not specified in the study report.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations were not performed in the study, as not required by the test guideline.

B. OBSERVATIONS

Systemic toxicity:

Mortality:
Four unscheduled deaths occurred during the course of the study. One female mated with a male of the 200 mg/kg bw group died in study week 3, one male of the 800 mg/kg bw group died in study week 5, one male of the 2000 mg/kg bw group died in week 6 and one female mated with a male of the 2000 mg/kg bw dose group died in study week 2. The cause of death could not be determined at necropsy. The observations on mortality were not attributed to treatment and considered to be incidental.

Clinical signs of toxicity:
There were no test item-related signs of systemic toxicity observed. Incidental findings included alopecia, matting of the hair coat and enlargement of the genital area. These findings were observed randomly throughout various weeks of the study among all treatment groups. Due to the random occurrence and small number of total animals affected, the findings were not related to treatment.

C. BODY WEIGHT AND BODY WEIGHT DEVELOPMENT OF SIRES

There were no biologically meaningful differences in body weight and body weight gain in any of the treatment groups when compared to control animals over the entire study period.

D. NECROPSY OF SIRES

Necropsy of unscheduled sires revealed no abnormal findings which were related to treatment.

E. FERTILITY INDICES

Treatment with glyphosate did not affect the number and percentage of pregnant females throughout the course of the study (refer to Table). In addition, the number of *corpora lutea* (refer to Table B.6.4.3.5-2B.6.4.3.5-2) and the number of implantations (refer to

) were not affected by treatment.

Table B.6.4.3.5-1: Summary of results obtained in the dominant lethal assay, number and percentage of pregnant females ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
No. of pregnant females (%)					
Mating study week					
1	17 (85)	15 (75)	16 (80)	17 (85)	15 (75)
2	17 (85)	16 (80)	16 (80)	18 (90)	18 (90)
3	14 (70)	18 (90)	18 (90)	17 (85)	16 (80)
4	19 (95)	13 (65)	17 (85)	17 (85)	14 (70)
5	20 (100)	12 (60)	17 (85)	18 (90)	18 (90)
6	18 (90)	14 (70)	19 (95)	17 (85)	15 (75)
7	19 (95)	19 (95)	18 (90)	15 (75)	12 (60)
8	18 (90)	14 (70)	18 (90)	20 (100)	16 (80)

PC: positive control Cytoxan

Table B.6.4.3.5-2: Summary of results obtained in the dominant lethal assay, number of *corpora lutea* ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
No. of corpora lutea: Mean per dam ± SD					
Mating study week					
1	12.9 ± 1.68	11.1 ± 2.27	11.8 ± 1.68	11.2 ± 2.17	11.5 ± 2.80
2	11.2 ± 2.70	11.4 ± 1.55	11.1 ± 2.49	11.4 ± 1.42	9.1 ± 3.44**
3	13.3 ± 1.38	14.4 ± 2.67	13.1 ± 1.51	12.3 ± 1.16	10.9 ± 2.25**
4	12.5 ± 2.41	11.9 ± 2.95	13.2 ± 2.35	12.0 ± 4.28	13.5 ± 1.05
5	13.3 ± 2.00	12.3 ± 1.57	12.2 ± 2.13	12.6 ± 1.54	13.1 ± 1.44
6	13.8 ± 1.77	13.9 ± 1.20	12.9 ± 1.83	14.1 ± 2.29	11.4 ± 1.50
7	13.5 ± 1.84	13.3 ± 3.18	13.2 ± 2.87	13.6 ± 1.80	11.8 ± 2.44
8	12.7 ± 1.45	14.1 ± 1.88	12.4 ± 3.32	13.9 ± 2.43	13.5 ± 1.87

PC: positive control Cytoxan

** $p < 0.01$ statistical significance when compared to controls

Table B.6.4.3.5-3: Summary of results obtained in the dominant lethal assay, number of implantation sites ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
No. of implantation sites: Mean per dam ± SD					
Mating study week					
1	12.5 ± 1.28	11.3 ± 2.74	11.6 ± 1.78	12.2 ± 2.43	9.1 ± 2.95
2	12.2 ± 2.30	12.5 ± 1.90	11.6 ± 1.89	13.1 ± 1.25	7.4 ± 3.18
3	12.6 ± 1.22	13.4 ± 1.46	12.2 ± 1.83	11.6 ± 1.17	9.8 ± 2.17
4	12.1 ± 2.31	11.6 ± 2.90	12.5 ± 2.48	10.8 ± 3.81	12.7 ± 1.44
5	12.5 ± 2.12	11.6 ± 2.27	12.0 ± 2.26	11.8 ± 1.90	12.3 ± 2.09
6	12.8 ± 2.36	13.4 ± 1.39	12.2 ± 2.16	12.3 ± 2.55	13.1 ± 1.44
7	13.2 ± 1.51	12.1 ± 2.55	11.8 ± 3.28	13.0 ± 1.96	11.4 ± 2.91
8	12.0 ± 2.06	12.9 ± 2.91	11.2 ± 2.98	11.9 ± 2.41	12.6 ± 1.91

PC: positive control Cytoxan

The amount of early and late resorptions was comparable to those of control animals for all mating intervals (refer to Tables below). There were no non-viable fetuses in any dose group of any mating interval. However, there was a slight but statistically significant decrease in the number of viable fetuses in females of the 800 mg/kg bw group mated during study week 1 and in females of the 2000 mg/kg bw group mated during study week 3 (refer to Table B.6.4.3.5-6). As no increase in early fetal deaths were observed in these groups, the findings were not attributed to glyphosate treatment and considered to be incidental.

Table B.6.4.3.5-4: Summary of results obtained in the dominant lethal assay, number of early resorptions ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
Early resorptions: Mean per dam					
Mating study week					
1	0.6 ± 0.93	0.7 ± 1.54	0.8 ± 1.11	0.4 ± 0.62	4.9 ± 2.12**
2	0.9 ± 2.03	1.0 ± 1.41	0.7 ± 1.46	0.8 ± 0.90	4.1 ± 1.89**
3	0.5 ± 0.76	1.3 ± 1.53	0.8 ± 1.00	0.6 ± 0.80	4.4 ± 3.18**
4	0.8 ± 1.27	0.6 ± 1.00	1.2 ± 1.67	0.9 ± 0.86	1.5 ± 2.07
5	0.4 ± 0.83	0.5 ± 0.72	1.3 ± 3.39	0.9 ± 1.28	0.5 ± 0.52
6	0.8 ± 1.48	0.6 ± 0.77	0.6 ± 0.79	0.9 ± 1.22	1.2 ± 2.01
7	1.3 ± 1.41	0.6 ± 0.90	0.4 ± 0.61	0.5 ± 0.64	1.0 ± 1.21
8	0.5 ± 0.99	0.9 ± 1.13	0.6 ± 0.76	1.1 ± 1.53	0.7 ± 1.59

PC: positive control Cytoxan

** $p < 0.01$ statistical significance when compared to controls

Table B.6.4.3.5-5: Summary of results obtained in the dominant lethal assay, number of late resorptions ([redacted], 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
Late resorptions: Mean per dam ± SD					
Mating study week					
1	0.0 ± 0.00	0.1 ± 0.35	0.3 ± 0.70	0.1 ± 0.33	0.1 ± 0.35
2	0.2 ± 0.33	0.1 ± 0.25	0.8 ± 2.81	0.2 ± 0.44	0.0 ± 0.00
3	0.1 ± 0.27	0.1 ± 0.24	0.1 ± 0.24	0.8 ± 3.15	0.6 ± 2.00
4	0.9 ± 3.21	0.2 ± 0.39	0.0 ± 0.00	0.0 ± 0.00	0.2 ± 0.60
5	0.8 ± 3.21	0.3 ± 0.59	0.1 ± 0.33	0.4 ± 0.78	0.1 ± 0.29
6	0.2 ± 0.55	0.2 ± 0.37	0.1 ± 0.33	0.1 ± 0.26	0.3 ± 0.61
7	0.2 ± 0.50	0.1 ± 0.23	0.3 ± 0.75	0.3 ± 0.59	0.2 ± 0.39
8	0.1 ± 0.32	1.3 ± 3.56	0.3 ± 0.44	0.6 ± 1.26	0.5 ± 1.87

PC: positive control Cytoxan

Table B.6.4.3.5-6: Summary of results obtained in the dominant lethal assay, number of viable fetuses ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
Viable fetuses: Mean per dam ± SD					
Mating study week					
1	11.8 ± 1.38	10.5 ± 3.58	10.5 ± 1.83*	11.6 ± 2.32	4.1 ± 3.77**
2	11.1 ± 3.35	11.4 ± 2.31	10.1 ± 3.26	12.1 ± 1.89	3.3 ± 3.34**
3	12.0 ± 1.30	12.0 ± 2.42	11.4 ± 2.15	10.3 ± 2.95*	4.8 ± 3.17**
4	10.4 ± 3.76	10.8 ± 3.24	11.3 ± 3.10	9.9 ± 4.46	10.9 ± 2.87
5	11.3 ± 3.27	10.8 ± 1.95	10.4 ± 4.09	10.4 ± 2.94	11.7 ± 2.06
6	11.8 ± 2.43	12.6 ± 1.42	11.4 ± 2.09	11.3 ± 3.04	11.6 ± 1.87
7	11.8 ± 1.84	11.4 ± 2.76	11.1 ± 3.05	12.3 ± 1.98	10.3 ± 3.08
8	11.4 ± 2.20	10.7 ± 4.25	10.4 ± 2.94	10.3 ± 2.91	11.4 ± 3.88

PC: positive control Cytoxan

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls

Further, the incidence of pre- and post-implantation losses (refer to Tables below) was not considered to be affected upon treatment with glyphosate and the retrospectively calculated dominant lethal factors (refer to Table B.6.4.3.5-9) were comparable for glyphosate treated and control animals.

Table B.6.4.3.5-7: Summary of results obtained in the dominant lethal assay, number of pre-implantation losses ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
Pre-implantation loss$: Mean per dam ± SD					
Mating study week					
1	0.47 ± 0.80	0.93 ± 2.15	0.19 ± 0.40	1.06 ± 1.30	2.33 ± 3.33
2	1.00 ± 1.12	0.94 ± 1.29	1.56 ± 1.85	0.35 ± 0.61	1.69 ± 2.39
3	0.71 ± 0.73	1.00 ± 2.15	0.83 ± 0.92	0.65 ± 0.79	1.13 ± 1.15
4	0.37 ± 0.68	0.35 ± 0.61	0.71 ± 0.77	1.12 ± 2.12	0.77 ± 0.73
5	0.84 ± 0.96	0.71 ± 1.49	0.18 ± 0.64	0.78 ± 1.11	0.83 ± 1.40
6	0.94 ± 1.55	0.53 ± 1.02	0.71 ± 0.92	1.80 ± 2.08	0.29 ± 0.61
7	0.32 ± 0.75	1.26 ± 2.31	1.39 ± 2.06	0.60 ± 0.74	0.42 ± 0.67
8	0.72 ± 1.67	1.22 ± 1.77	1.20 ± 1.11	2.00 ± 2.58	0.86 ± 1.70

PC: positive control Cytoxan

$: Calculated based on raw data provided in study report as follows: (number of corpora lutea - number of implants)/number of pregnant females

*: $p < 0.05$ and ** $p < 0.01$ statistical significance when compared to controls

Table B.6.4.3.5-8: Summary of results obtained in the dominant lethal assay, number of post-implantation losses ([redacted] 1980)

	Group				
	1	2	3	4	PC
	Dose [mg/kg bw]				
	0	200	800	2000	240
Post-implantation loss: Mean per dam ± SD					
Mating study week					
1	0.6 ± 0.93	0.8 ± 1.52	1.1 ± 1.20	0.5 ± 0.72	5.1 ± 2.12**
2	1.1 ± 2.05	1.1 ± 1.39	1.5 ± 3.22	1.0 ± 1.06	4.1 ± 1.89**
3	0.6 ± 0.76	1.4 ± 1.50	0.8 ± 0.99	1.4 ± 3.10	4.9 ± 3.36**
4	1.7 ± 3.35	0.8 ± 1.03	1.2 ± 1.67	0.9 ± 0.86	1.6 ± 2.42
5	1.2 ± 3.25	0.8 ± 0.88	1.6 ± 3.47	1.3 ± 1.88	0.6 ± 0.51
6	1.0 ± 1.36	0.7 ± 0.99	0.8 ± 0.75	1.0 ± 1.20	1.5 ± 1.91
7	1.4 ± 1.54	0.6 ± 0.90	0.7 ± 0.97	0.7 ± 0.70	1.2 ± 1.19
8	0.6 ± 0.98	2.2 ± 3.47	0.8 ± 1.01	1.6 ± 2.19	1.2 ± 2.52

PC: positive control Cytoxan

Table B.6.4.3.5-8: Summary of results obtained in the dominant lethal assay, number of post-implantation losses ([redacted] 1980)

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw]				
	0	**200**	**800**	**2000**	**240**

** $p < 0.01$ statistical significance when compared to controls

Treatment with the positive control Cytoxan® induced statistically significant dominant lethal effects and effect at the postmeiotic stage of spermatogenesis and demonstrated the validity of the test. When compared to the vehicle control group, there was a statistically significant decrease in the number of viable fetuses and an increase in the proportion of early fetal deaths, indicated by a statistically significant increase in the mean number of early resorptions and post-implantation loss observed during the first 3 weeks of mating.

Table B.6.4.3.5-9: Summary of results obtained in the dominant lethal assay, dominant lethal factors ([redacted] 1980)

	Group				
	1	**2**	**3**	**4**	**PC**
	Dose [mg/kg bw]				
	0	**200**	**800**	**2000**	**240**
Dominant lethal factor[#]					
Mating study week					
1	5.19	7.10	9.68	4.35	55.47
2	8.70	8.50	12.98	7.66	55.46
3	4.55	10.13	6.82	11.62	50.64
4	14.35	6.60	9.43	7.95	13.94
5	9.28	7.11	13.24	11.32	4.76
6	8.23	5.51	6.28	8.11	11.48
7	10.76	5.24	5.66	5.64	10.22
8	5.09	16.81	7.17	13.61	9.60

PC: positive control Cytoxan

[#]: Calculated as follows: (total post implantation loss per female / total implantations per female) x 100

In addition, there was a lower number of implantations observed during the first three weeks of mating after treatment with Cytoxan® (refer to

), with a statistically significant decrease at mating weeks 2 and 3 when compared to control animals (refer to Table). The observations were attributed to a decreased ovulation rate, as seen by a lower number of corpora lutea during these weeks (refer to Table B.6.4.3.5-2).

III. CONCLUSION

Under the conditions of the test, glyphosate had no mutagenic potential in CD-1 mice in the dominant lethal assay up to a dose level of 2000 mg/kg bw. Based on the experimental findings, glyphosate was considered non-genotoxic to germ cells *in vivo*.

Assessment and conclusion by applicant:

Negative for mutagenic effects in the dominant lethal assay in mice.

The experimental performance of the study was not conducted under GLP criteria, but matched the descriptions of OECD guideline 478 (2016). However, there was a large number of deviations when compared to the current guideline. The number of implantations per group for each mating period was below the recommended number of 400 implants per group. It can be assumed that the number of males and females used for mating was too low. Fetal body weights were not recorded and no historical control data were provided. No dominant lethal frequency was calculated in the study report, but as the raw data were provided in the study report, dominant lethal factors could be calculated retrospectively for this evaluation. Further deviations were of minor degree and considered to not compromise the outcome of the study. Due to the large number of deviations, the study was considered to provide supporting information.

Assessment and conclusion by RMS: It is agreed with the applicant's conclusion. Glyphosate was negative for mutagenic effects in the dominant lethal assay in mice under the conditions of this test. None of the observations was dose-related or consistent over the mating periods. The calculations of the total post implantation loss per female / total implantations per female) x 100 could be recalculated.
The study is considered supportive with restrictions due to the noted deviations.

B.6.4.4. Genotoxicity – Information from public literature

B.6.4.4.1. *Genotoxicity – public literature, study 1*

Data point:	CA 5.4/001
Report author	Adler-Flindt, S., Martin, S.
Report year	2019
Report title	Comparative cytotoxicity of plant protection products and their active ingredients
Document No	doi.org/10.1016/j.tiv.2018.10.020 ISSN: 0887-2333
Guidelines followed in study	Not applicable
Deviations from current test guideline	Not applicable
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered reliable with restrictions (Klimisch score 2).

Full summary of the study according to OECD format

In this study plant protection products (PPPs) were analyzed for the correlation of GHS classifications resulting mainly from in vivo LD50-values with classifications obtained from calculated LD50-values using the CLP calculation method (CM). Accordingly, the CM predicted 80 % of the PPPs correctly. However 31 % of classified products were not identified revealing a considerable inaccuracy of this method. Based on these results ten PPPs and corresponding active substances (ASs) were further tested in a cytotoxicity assay employing 3T3 and hFF cells (one PPP and corresponding AS were tested in HepaRG cells).

Materials and methods

Chemicals - Glyphosate isopropylamine salt (MON 0138; CAS 38641-94-0) and RoundUP LB Plus (German registration number 024142-60; 360 g/L glyphosate equivalents) was purchased from Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany. Glyphosate (IPA salt) was received already dissolved in water at a concentration of 620 g/L.

Culture of Balb/3T3 cells, hFF cells and HepaRG cells – Mouse fibroblast cells (Balb/3T3) and human foreskin fibroblast cells (hFF) were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum (FBS) and 50 U/mL penicillin/streptomycin, and passaged every 3 to 4 days at a split-ratio of approximately 1:20 for 3T3 cells and 1:8 for hFF cells after enzymatic dissociation with trypsin-EDTA. Differentiated HepaRG cells were received in 96-well plates and were grown for 2 weeks in Williams's medium containing 10 % foetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.05 % human insulin and 50 µM hydrocortisone hemisuccinate. For differentiation, cells were incubated for a another 2 weeks in differentiation medium containing in addition to the above components 1.7 % DMSO.

Proliferation test - To determine the optimal seeding density for the 48-hour toxicity assay, proliferation tests were performed in 2 independent runs. 3T3 and hFF cells were seeded in test medium (DMEM) supplemented with 10 % (v/v) Panexin NTA serum substitute and 50 U/mL penicillin/streptomycin, into 96-well plates, in a 2-fold dilution series, with the highest cell density being 1.6×10^4 cells/well. After 48 hours the viability of the cells was assessed by measuring the reduction of resazurin to the fluorescent resorufin. The fluorescence signal was measured at 530 nm (excitation) and at 590 nm (emission) using a multimode plate reader.

Cytotoxicity test - 3T3 and hFF cells were dissociated into single cells and seeded into 96-well plates in 100 µL per well of DMEM supplemented with 10 % (v/v) Panexin NTA serum substitute and 50 U/mL penicillin/streptomycin, at a density of 2,000 cells per well for 3T3 cells and 4,000 cells per well for hFF cells. Differentiated HepaRG cells were received in 96-well plates already seeded in a density of 9,000 cells per well. The test medium for HepaRG cells was based on phenol-red-free Williams's medium containing 2 % foetal calf serum (FCS), 100 U/mL penicillin/streptomycin, 0.05 % human insulin and 50 µM hydrocortisone hemisuccinate. After 24 hours, 100 µL of test medium (containing the double required final concentration of the test substance) were added to each well (day 0). After 48 hours cell viability was assessed by measuring the reduction of resazurin to the fluorescent resorufin. The fluorescence signal was measured at 530 nm (excitation) and at 590 nm (emission) using a multimode plate reader.

Testing of glyphosate and RoundUP LB Plus - At least two independent runs of each experiment were performed. The highest tested concentration for glyphosate isopropylamine salt was 1000 µg/mL. RoundUP LB Plus was tested in concentrations resulting in the same concentration as the active substance.

Results

Proliferation assay - Proliferation tests were performed to determine the optimal seeding density for the 48-hour toxicity assay. The cells were seeded in a 2-fold dilution series into 96-well plates, and reduction of resazurin into the fluorescent resorufin was measured after 48 hours. The proliferation assay revealed an optimal seeding concentration for the cytotoxicity test of 2,000 cells per well for 3T3 cells and 4,000 cells per well for hFF cells.

Testing of glyphosate and RoundUP LB Plus - Two controls, saccharin as the negative control and 5-FU as the positive control, were tested for 48 hours on 3T3 and hFF cells. The treatment of both cell types with saccharin did not significantly reduce cell viability up to a concentration of 1,000 µg/mL. In contrast, the treatment with 5-FU resulted in a noticeable reduction of viability, with different IC_{50} values for the two cell types, i.e. 0.06 ± 0.01 µg/mL for 3T3 cells and 0.14 ± 0.05 µg/mL for hFF cells. The IC_{50} for glyphosate isopropylamine salt was 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells. The IC_{50} for RoundUP LB Plus was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The ratio of the AUC under the % viability vs concentration curve of glyphosate isopropylamine salt over RoundUP LB Plus is 1.7 for 3T3 cells and 1.3 for hFF cells. This indicates that the treatment of hFF cells with glyphosate and its formulation Roundup did not result in significant differences between cytotoxicity curves. The ratio of the AUCs of glyphosate over RoundUP LB Plus for both cell types was below a factor 2 and could thus be regarded as minor.

Conclusion

In this study, glyphosate isopropylamine salt, amongst other pesticides, and its corresponding formulation RoundUP LB Plus were tested for cytotoxicity in 3T3 cells and hFF cells. The IC_{50} for glyphosate isopropylamine salt was 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells and the IC_{50} for RoundUP LB Plus was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The ratio of the AUCs of glyphosate over MON 52276 for both cell types was below a factor 2 and could thus be regarded as minor.

Assessment and conclusion by applicant:

It was the intention of this study to evaluate the GHS classification of pesticide formulations for acute toxicity based on calculated LD_{50} values using the CLP calculation method (CM). Because of the considerable inaccuracy of this method the *in vitro* cytotoxicity of 10 pesticide formulations was compared against that of the active ingredient using mouse (3T3) and human (hFF) fibroblasts. In this exercise the IC_{50} for glyphosate isopropylamine salt was found to be 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells and the IC_{50} for MON 52276 was 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells. The difference in cytotoxicity (expressed as the AUC of the % viability vs concentration curve) between glyphosate and MON 52276 could be regarded as minor.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate isopropylamine salt used was not sufficiently characterized and the standard deviation of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is too large.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Adler-Flindt *et al.*, 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Glyphosate isopropylamine salt,

		purity not reported. Source: Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany.
Only glyphosate acid or one of its salts is the tested substance	N	MON 52276 (RoundUP LB Plus, 360 g/L A.I.). Source : Monsanto Agrar Deutschland GmbH, Düsseldorf, Germany. Other pesticides and their formulations were tested as well.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Mouse (3T3) and human (hFF) fibroblasts.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (for local contact)	Test concentrations up to 1000 µg/mL which is beyond the systemic physiological range but not when applied dermally.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	Saccharin was used as the negative control and 5-FU as the positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	IC_{50} were calculated.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate isopropylamine salt used was not sufficiently characterized and the standard deviation of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is too large.		

Assessment and conclusion by RMS:

In this study, the cytotoxicity of glyphosate (as IPA salt) and the product RoundUP LB Plus (360 g/l glyphosate eq.) was tested in murine (3T3) and human (hFF) fibroblasts. Cytotoxicity was determined as the half maximal inhibitory concentration (IC_{50}). The IC_{50} for glyphosate isopropylamine salt was found to be 954.8 ± 117.1 µg/mL for 3T3 cells and 1211 ± 885.7 µg/mL for hFF cells and the IC_{50} for RoundUP LB Plus was found to be 313.2 ± 29.3 µg/mL for 3T3 cells and 361.6 ± 612 µg/mL for hFF cells.

Furthermore, the difference in cytotoxicity (expressed as the AUC of the % viability vs concentration curve) between glyphosate and RoundUP LB Plus was investigated. The AUCs were regarded as more appropriate for comparison than the IC_{50} values by the study authors because they take the whole course of the curves into account instead of just single points on the curves. No significant difference between the cytotoxicity curves for glyphosate (IPA salt) and RoundUP LB Plus was observed.

As already indicated by the applicant, the standard deviations of the IC_{50} of glyphosate (1211 ± 885.7 ug/mL) and MON 52276 (361.6 ± 612 µg/mL) for human fibroblasts is relatively high so that solid conclusions cannot be made.

The RMS agrees with the applicant conclusion that this study is reliable with restrictions.

It is, however, noted that in this study genotoxicity is not investigated, nor is the cytotoxicity discussed in relation to genotoxicity. The study is presented in this section for practical reasons.

B.6.4.4.2. *Genotoxicity – public literature, study 2*

Data point:	CA 5.4/002
Report author	Ilyushina, N. *et al.*
Report year	2018a
Report title	Maximum tolerated doses and erythropoiesis effects in the mouse bone marrow by 79 pesticides' technical materials assessed with the micronucleus assay
Document No	doi.org/10.1016/j.toxrep.2018.12.006 ISSN: 2214-7500
Guidelines followed in study	Not applicable (although the study setup is similar to OECD Guideline 474)
Deviations from current test guideline OECD 474 (2016)	Positive control animals were included but the data are not reported. No ratio of PCE to NCE was reported. Data have been presented per group rather than per animal. HCD data not included.
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive due to the deviations noted above.

Full summary of the study according to OECD format

Effects of technical materials of pesticide active ingredients, belonging to various chemical classes, on erythropoiesis in mouse bone marrow were studied as part of the research on the pesticide mutagenic activity in micronucleus test. The purpose of the present study was to estimate the toxic action of the test substances on the target organ and the validity of the results of the micronucleus assay under conditions of erythropoiesis suppression.

Materials and methods
Chemicals - Four glyphosate batches were tested with a purity of respectively 95.7, 98.3, 95.1, and 95.8 %.

Animals – CD-1 mice were purchased from "Andreevka" Branch of the Federal Government Budgetary Establishment of Science "Scientific Center of Biomedical Technologies" of the Federal Bio-Medical Agency of the Russian Federation. The acclimation period was 7 days. Mice had access to drinking water and feed ad libitum, and were maintained under a 12:12-hour light/dark photoperiod at 22 – 22.5 °C and 36 - 40 % humidity.

Mammalian Erythrocyte Micronucleus Test (OECD TG 474) - At least 5 groups of minimum 5 mice per sex were used. Each assay included a positive control group (40 mg/kg bw cyclophosphamide), a negative control group (1 % potato starch in water), and 3 treatment groups. Glyphosate was administered orally by gavage once a day for 2 subsequent days (24 hours apart) at a volume of 10 mL/kg bw. The maximum dose in the main experiment was 2,000 mg/kg bw. Mice were sacrificed 22 hours after the second administration by cervical dislocation, then femurs were removed and bone marrow was harvested. Bone marrow smears were prepared on microscope slides (2 slides per animal), air-dried, fixed, stained with azure-eosin, and independently coded by a researcher not engaged in the cell counting process. To assess the effect of glyphosate on erythropoiesis, the ratio of polychromatic erythrocytes (PCEs) to the sum of PCEs and normochromatic erythrocytes (NCEs) was determined by counting at least 500 cells (PCEs + NCEs) per animal (at least 250 cells for each slide) under a Nikon Eclipse Ci-L microscope. At least 4,000 PCEs were counted per animal by two different researchers.

Statistical analysis - Statistical analysis was performed using SPSS Statistics v. 22.0 software. The statistical significance of the difference in the proportion of PCEs/(PCEs + NCEs) between the highest dose group and the concurrent negative control group was evaluated using the independent samples t-test for each study.

Results
It should be noted that the negative control values slightly varied from experiment to experiment, and that the historical negative controls of the laboratory were 0.50 ± 0.06 and 0.52 ± 0.06 PCEs/(PCEs+NCEs) females and males, respectively. Cyclophosphamide did not cause a significant decrease in the proportion PCEs/(PCEs+NCEs) in comparison with the negative control. The 4 glyphosate batches tested at the limit dose of 2,000 mg/kg bw in CD-1 mice according to the protocol of the *in vivo* micronucleus assay compliant with OECD test guideline 474 did not reveal any effect on erythropoiesis in the bone marrow.

Conclusion
Glyphosate tested at the limit dose of 2,000 mg/kg bw in mice in the *in vivo* micronucleus assay did not show any effect on erythropoiesis in the bone marrow.

Assessment and conclusion by applicant:
Along with 51 other pesticides 4 batches of glyphosate with purities ranging from 95.1 to 98.3 % were investigated for their effect on erythropoiesis in mice. To assess the toxicity of glyphosate on the bone marrow the *in vivo* micronucleus test in the mouse according to OECD test guideline 474 was conducted at the limit dose of 2,000 mg/kg bw. No effect of glyphosate on erythropoiesis was found.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the glyphosate batches used was not reported and no suitable positive control was used in the micronucleus test. The test conducted was in compliance with OECD test guideline 474.

Reliability criteria for *in vivo* toxicology studies made by the applicant

Publication: Ilyushina *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD TG 474, in vivo MN assay only used with the purpose to assess toxicity to erythropoiesis in the bone marrow.
Study performed according to GLP	N	Not stated.
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y?	4 batches were tested with purity of 95.7, 98.3, 95.1, and 95.8 %. Source was not mentioned.
Only glyphosate acid or one of its salts is the tested substance	N	51 other pesticides were tested as well.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	
Dose levels reported	Y	Only limit dose of 2,000 mg/kg bw was considered.
Number of animals used per dose level reported	Y	At least 5 groups of minimum 5 mice per sex.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	Only effect on erythropoiesis was reported.
Statistical methods described	Y	
Historical control data of the laboratory reported	Y	
Dose-effect relationship reported	N	Not possible since only one dose was used.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the glyphosate batches used was not reported. The test conducted was in compliance with OECD test guideline 474.		

Assessment and conclusion by RMS:

Next to various other active substances, four batches of glyphosate with purities ranging from 95.1 to 98.3% were investigated for their effect on erythropoiesis in mice. This *in vivo* micronucleus test in the mouse was conducted largely according to OECD Guideline 474 at the limit dose of 2000 mg/kg bw. No effect of glyphosate on erythropoiesis was found for any of the four batches of glyphosate.

It is noted, however, that the description of the results of the experiments with glyphosate are very limited in the publication. As already indicated by the applicant, the source of the active substance is unknown. Further, it is not demonstrated that the target tissue, i.e. bone marrow, was exposed.

Overall, it is agreed with the applicant that the study is considered reliable with restrictions (source of glyphosate batches not reported; limited detail on methodology)

B.6.4.4.3. *Genotoxicity – public literature, study 3*

Data point:	CA 5.4/003
Report author	Nagy, K. *et al.*
Report year	2019
Report title	Comparative cyto- and genotoxicity assessment of glyphosate and glyphosate-based herbicides in human peripheral white blood cells
Document No	doi.org/10.1016/j.envres.2019.108851 E-ISSN: 1096-0953
Guidelines followed in study	None (OECD GL 489 only describes *in vivo* comet assays, whereas an *in vitro* comet assay was performed within this publication; the comet assay itself, however, was conducted similarly to the procedure described in OECD GL 489)
Deviations from current test guideline	– OECD GL 489 states that 2 or 3 slides per sample should be scored when five animal per group are used, whereas 2 x 50 randomly captured comets from duplicate slides were scored in the current publication. Therefore, the number of scored slides is not sufficient when compared to the OECD GL requirements. – No HCD are available. Furthermore, although the publication states that hydrogen peroxide was used as positive control, results of the positive control are not shown. Therefore, the proficiency of the research group cannot be proven. – Test substance characteristics (purity, batch, expiration date etc.) are not included in the publication. – Stability of test substance and concentration of tested concentrations not analytically verified
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive due to the deviations mentioned above.

Full summary of the study according to OECD format

This study investigates the cyto- and genotoxic potential of the active ingredient glyphosate and glyphosate-based herbicides (GBHs) in human mononuclear white blood (HMWB) cells. HMWB cells were treated for 4 h at 37 °C with increasing concentrations (1–1000 μM) of glyphosate alone and in three GBHs (Roundup Mega, Fozat 480 and Glyfos) to test cytotoxic effect with fluorescent co-labelling and genotoxic effect with comet assay. In addition, each concentration was tested with and without metabolic activation using human liver S9 fraction.

Materials and methods

Chemicals; Analytical-grade glyphosate (N-(phosphonomethyl) glycine, CAS No: 1071-83-6) was purchased from VWR International Kft (Debrecen, Hungary) and samples of three GBHs, i.e.

- Roundup Mega containing 551 g/L or 42 % (w/w) potassium salt of glyphosate (CAS No: 70901-12-1; equivalent to 450 g/L glyphosate) and 7 % (w/w) ethoxylated etheralkylamine (CAS No: 68478-96-6);
- Fozat 480 containing 480 g/L or 41 % (w/w) isopropylammonium salt of glyphosate (CAS No: 38641-94-0; equivalent to 360 g/L glyphosate) and < 5 % (w/w) hygroscopic substances;
- Glyfos containing 480 g/L or 42 % (w/w) isopropylammonium salt of glyphosate (equivalent to 360 g/L glyphosate) and 9 % (w/w) polyethoxylated tallow amine (CAS No: 61791-26-2);

were kindly provided by pesticide applicators. Composition data for each formulation were retrieved from the material safety data sheets (MSDS). Chemicals used for the assays and human liver-derived metabolic activation system (S9 fraction) were obtained from Sigma-Aldrich Chemie GmbH (Heidelberg, Germany). Cell culture medium and its supplements were obtained from Biowest (Nuaillè, France). The acetomethoxy derivative of calcein (Calcein AM) and propodium iodide (PI) fluorescent dyes were purchased from Biotium (Hayward, CA, USA). Heparin-containing vacutainers were purchased from BD Vacutainer Systems (Plymouth, UK).

Cell cultures; Human peripheral whole blood samples were obtained by venipuncture and collected into heparin-containing vacutainer tubes from four non-smoking, healthy volunteers (three males and one female, aged 20–40 years) without known previous contact with pesticides, mutagens or carcinogens. Cultures were prepared within 1 h of phlebotomy. Human mononuclear white blood (HMWB) cells were prepared from erythrocytes by density-gradient centrifugation over Histopaque-1077 gradient. The buffy coat was aspirated and re-suspended in RPMI 1640 medium containing 10 % foetal calf serum (FCS).

Cell treatment; HMWB cells were treated in the cell-culture medium with increasing concentrations (1 μM, 10 μM, 100 μM, 250 μM, 500 μM, 750 μM and 1 mM) of glyphosate alone and in three GBHs in a way that the concentrations of glyphosate in GBHs were equivalent. Concentrations were chosen based on the results from previous in vitro studies performed on human lymphocytes. The stock solutions and the dilution series were made in phosphate-buffered saline (PBS) and adjusted with 1 M NaOH to pH 7.2. Aliquots of different concentrations of glyphosate and GBH solutions, as well as PBS as negative control, were added to the cell cultures and incubated for 4 h at 37 °C. The PBS content was always < 10 % (v/v) in the cell culture medium. The experiments were conducted in the presence and absence of S9 fraction. 100 μL of the working S9 mix containing 10 % (v/v) of S9 fraction was composed of 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP was added to the S9+ samples. 100 μM hydrogen peroxide was used as a positive control.

Cytotoxicity assay; After treatment, aliquots of samples were immediately subjected to cytotoxicity test. Calcein AM and propidium iodide (PI) fluorescent dyes were used to co-label the cells. Calcein AM is a non-polar compound that passively crosses the plasma membrane of living cells, where it is cleaved by intracellular esterases to reveal a very polar derivative of fluorescein (calcein) that remains trapped in the cytoplasm. PI is a DNA intercalating dye, which is able to permeate membranes of dead and dying cells but cannot penetrate plasma membranes of live healthy cells. Both fluorescent dyes were dissolved in PBS (pH 7.2) to a final concentration of 2 μM each. 200 μL of this working solution added to the cell pellets (1×10^5 cells), and incubated for 30 min at 4 °C, protected from light. The labelled cells were washed and re-suspended in ice-cold PBS buffer. 40 μL of the cell suspension was put on a microscope slide for immediate microscopic examination at 100x magnification using a Zeiss Axioplan epifluorescent microscope. FITC filter for Calcein AM and TRITC filter for PI was applied to excite the co-labelled cells. Survival rate was determined by visual examination of 10 randomly selected non-overlapping fields per slide. Each field contained 10 to 30 images. Cell viability was expressed as the mean of the percentages of living cells from repeated experiments. The proportions of living cells observed in technical replicates were subjected to statistical analysis.

Genotoxicity assay; The alkaline version of the comet assay was performed according to the methodology of Collins (2004). Following treatment, samples were centrifuged and HMWB cells were resuspended in serum-free medium at a cell density of 2000 cells/µL. Degreased frosted slides were coated with two layers: 1 % normal melting point agarose (NMA) covered with 0.75 % low melting point agarose (LMA) containing the cells (~2×10^5 per slide). After solidification, the embedded cells were lysed (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base, pH 10, 1 % sodium N-lauroyl sarcosinate and 1 % Triton X-100 added fresh) at 4 °C for 18–20 h, shielded from light. After lysis, the DNA was allowed to unwind for 20 min in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13) and subjected to electrophoresis in the same buffer for 20 min at 0.8 V/cm and 300 mA in a horizontal electrophoresis tank (Cleaver Scientific, Rugby, UK). Finally, the slides were rinsed gently three times with neutralization buffer (0.4 M Tris base-HCl, pH 7.5) to remove excess alkali and detergent. After drying, each slide was stained with ethidium bromide (20 µg/ml) and stored in a humidified container at 4 °C until analysis. The fluorescence signal was detected at 400x magnification using a Zeiss Axioplan epifluorescent microscope equipped with a CCD camera connected to an image analysis system. The Comet Imager v.2.2.1. Software (MetaSystems GmbH, Germany) was used to analyse 2 × 50 randomly captured comets from duplicate slides and compute the DNA damage parameters. Percentage of DNA in the tail (tail DNA%) and tail length in µm (TL) were measured to quantify DNA damage. The results are presented as mean of the median values of DNA damage parameters from repeated experiments. The medians of technical replicates were subjected to statistical analysis.

Data analysis; Experiments were independently performed three times from three different donors. The rate of cell viability and the central values of DNA damage parameters in the comet assay induced by various concentrations of the xenobiotics in repeated experiments were statistically compared to that of untreated cells using ANOVA with Dunnett's post hoc test. To statistically analyse the effect of metabolic activation, DNA damage values were pooled from three repeated experiments to compare results from S9-treated and S9-untreated samples at each exposure concentration by using Mann-Whitney test because pooled data sets followed non-normal distribution. Statistically significant difference was accepted at 5 % significance level.

Results

Cytotoxicity; The viability of HMWB cells treated with glyphosate alone was found to be over 86 % in the absence and presence of S9 over the entire concentration range (please refer to Figure below). Regardless of metabolic activation, GBHs induced a significant decrease in the proportion of living cells from 250 µM of Round Mega and Glyfos whereas from 500 µM of Fozat 480.

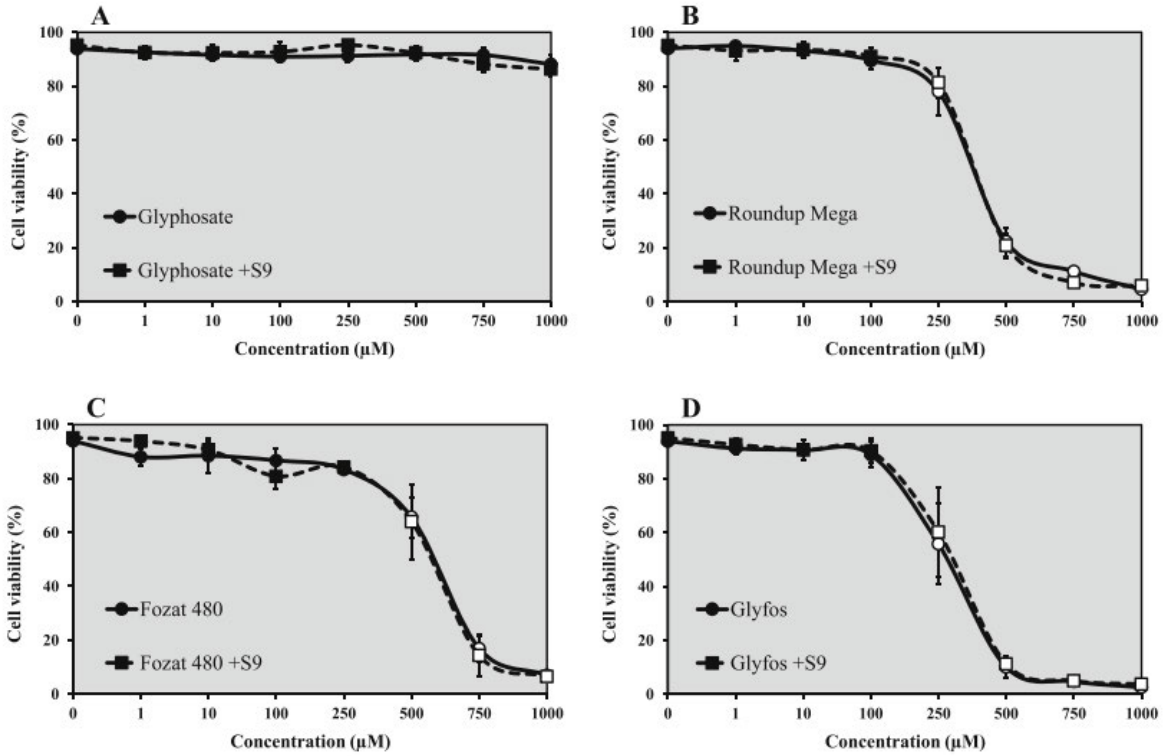


Figure B.6.4.4.3-1: Effect of 4-h exposure to increasing concentrations of glyphosate (A), Roundup Mega (B), Foza 480 (C) and Glyfos (D) on cell viability in the absence and presence (+S9) of metabolic activation system. The data points indicate the means ± standard error of the mean (SEM) and three repeated experiments. Statistically significant decrease of cell viability, indicated by empty data points, was determined by comparing the values induced by various doses of glyphosate or GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test. (from: Nagy *et al*., 2019)

DNA damage; Exposure of HMWB cells to glyphosate in the 0–1000 µM concentration range did not result in dose-dependent increase of DNA damage measured by the comet assay parameters. Unlike the active principle, GBHs induced statistically significant increase of both DNA damage parameters from 500 µM (Roundup Mega and Glyfos) and 750 µM (Fozat 480) compared to untreated cells. S9 treatment did not influence the effect of active ingredient glyphosate over the whole concentration range. On the contrary, addition of S9 to the assay modified the effects of GBHs in a non-consistent manner. The presence of metabolic enzymes significantly decreased the DNA damage induced by Roundup Mega and Fozat 480 at 1000 µM and from 10 µM, respectively. Metabolic activation could be observed only in samples exposed to 250 µM or higher concentrations of Glyfos indicated by the statistically significant differences in the tail DNA% and TL values between the S9-treated and S9-untreated cells (Tables 1 and 2).

Table B.6.4.4.3-1: DNA damage induced by 4-hour exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail DNA% (from: Nagy *et al*., 2019)

Concentration (µM)	Tail DNA% ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	1.51 ± 0.05				1.39 ± 0.18			
1	2.81 ± 0.30	2.61 ± 0.39	2.27 ± 0.60	1.51 ± 0.41	2.90 ± 0.49	1.91 ± 0.60	1.60 ± 0.05	3.64 ± 2.33††
10	2.86 ± 0.28	2.97 ± 0.87	8.39 ± 5.37	2.08 ± 0.69	2.03 ± 0.38	1.77 ± 0.82	2.19 ± 1.10†††	0.99 ± 0.10†††
100	2.51 ± 0.52	2.49 ± 0.94	13.09 ± 9.67	1.87 ± 0.80	2.06 ± 0.57	2.23 ± 0.77	1.99 ± 0.76†††	1.24 ± 0.33
250	2.42 ± 0.18	(3.47 ± 0.55)	6.59 ± 1.52	(6.11 ± 2.58)	3.01 ± 0.45	(3.58 ± 0.07)	2.97 ± 0.47†††	(22.84 ± 14.24†††)
500	2.60 ± 0.84	(16.88 ± 3.54***)	(7.47 ± 0.69)	(17.69 ± 2.06*)	2.98 ± 0.24	(13.71 ± 3.09)	(4.59 ± 0.65†††)	(36.81 ± 15.40†††
750	3.64 ± 1.00	(24.67 ± 2.56***)	(28.58 ± 1.93*)	(37.81 ± 5.92***)	3.46 ± 0.67	(25.64 ± 6.53**)	(24.20 ± 1.20***†	(50.47 ± 10.64*†††)
1000	1.57 ± 0.24	(44.75 ± 0.46***)	(45.94 ± 3.74***)	(54.58 ± 2.74***)	1.51 ± 0.16	(31.45 ± 4.14***†††)	(22.28 ± 2.40***†††)	(62.38 ± 2.99**†††)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant (†p < 0.05, ††p < 0.01, †††p < 0.001) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Table B.6.4.4.3-2: DNA damage induced by 4-hour exposure to glyphosate and GBHs with and without metabolic activation system (S9) in HMWB cells measured as tail length (µm) (from: Nagy *et al*., 2019)

Concentration (µM)	Tail length (µm) ± SEM							
	- S9				+ S9			
	Glyphosate	Roundup Mega	Fozat 480	Glyfos	Glyphosate	Roundup Mega	Fozat 480	Glyfos
0	0.47 ± 0.19				0.35 ± 0.06			
1	0.66 ± 0.06	0.52 ± 0.08	0.39 ± 0.08	0.11 ± 0.08	0.60 ± 0.08	0.34 ± 0.14	0.18 ± 0.04	0.56 ± 0.45††
10	0.67 ± 0.07	0.77 ± 0.27	1.77 ± 1.09	0.29 ± 0.15	0.65 ± 0.12	0.40 ± 0.17	0.57 ± 0.26†††	0.05 ± 0.04†††
100	0.64 ± 0.13	0.56 ± 0.27	2.70 ± 1.92	0.27 ± 0.15	0.68 ± 0.15	0.42 ± 0.18	0.41 ± 0.17†††	0.17 ± 0.10
250	0.99 ± 0.22	(1.09 ± 0.14)	1.51 ± 0.12	(1.27 ± 0.38)	0.90 ± 0.25	(1.03 ± 0.25)	1.00 ± 0.32†††	(4.41 ± 2.42†††)
500	0.93 ± 0.35	(3.93 ± 1.41*)	(1.62 ± 0.12)	(5.26 ± 1.37**)	0.81 ± 0.20	(2.87 ± 0.37*)	(1.19 ± 0.23†††)	(7.31 ± 2.27*†††)
750	1.05 ± 0.22	(5.40 ± 0.76**)	(6.57 ± 0.11**)	(8.91 ± 0.97***)	0.99 ± 0.21	(4.51 ± 1.07***)	(4.61 ± 0.19***†††)	(9.55 ± 0.50**†)
1000	0.31 ± 0.08	(6.77 ± 0.41***)	(8.13 ± 0.61**)	(10.09 ± 0.39***)	0.28 ± 0.07	(5.62 ± 0.32***†††)	(3.90 ± 0.31***†††)	(10.41 ± 0.22***†)

Data are means of median values of three repeated experiments.
Data in parentheses refer to samples with significant cytotoxic response.
Statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001) increase in DNA damage was determined by comparing the values of DNA damage induced by various doses of glyphosate and GBHs to the background level of untreated cells by ANOVA with Dunnett's post hoc test.
Statistically significant (†p < 0.05, ††p < 0.01, †††p < 0.001) difference in DNA damage levels between S9-treated and S9-untreated cells induced by the same concentration of glyphosate and GBHs was determined by Mann-Whitney test.

Discussion

The use of glyphosate containing products both for agricultural and residential purposes continues to rise since their first introduction to the market in 1974. Today, uncountable GBHs are registered in more than 130 countries worldwide. The unrestricted application of GBHs in the past few decades has resulted in the accumulation of glyphosate residues in environmental media exposing – and also impairing the health of – aquatic and terrestrial animals, as well as humans. In this study, we measured potential differences in the cyto- and genotoxicity between the declared active ingredient glyphosate and three marketed GBHs in HMWB cells in vitro with the use of the comet assay. To examine the toxicity of potential metabolites of the selected herbicides, human liver-derived metabolic enzyme system (S9) was also applied. The cytogenetic side effects of glyphosate and GBHs in humans have received pronounced scientific coverage in the last decade, resulting in numerous in vivo and in vitro human studies, which demonstrated that glyphosate alone or GBHs have detectable genotoxic potential, while others have reached opposite conclusions. Our results indicate that glyphosate alone could not induce DNA strand breaks in HMWB cells up to 1000 μM measured with the comet assay even in the presence of metabolic activation. This finding contrasts with previous studies employing isolated human blood mononuclear cells treated with glyphosate concentrations corresponding to ours, but using different experimental settings. A study showed with comet assay that glyphosate at 0.7 μM (in vitro) caused a statistically significant increase of DNA damage in human peripheral blood lymphocytes; however, they applied longer (20 h) exposure time than used in this study, and, unlike in our experiment, cells were incubated on microscope slides embedded in agarose gel at 25 °C. A further study also observed DNA damage in the above cell type from 20.7 μM (3.5 μg/ml) and from 250 μM, respectively. Although they incubated the cell cultures under the same conditions as used in our study (for 4 h at 37 °C), they applied LMA at lower concentration (0.5 %), as well as lower electrophoretic voltage (0.7 V/cm) and time (15 min). The execution of comet assay by them applying 24 h exposure time and lower electrophoretic voltage was also different from the method we used. These discrepancies in the experimental design along with the possible inter individual variability in response to genotoxic insults may explain the contradictory findings. The cytotoxic potential of the active ingredient glyphosate in the 0–1000 μM concentration range was found to be minimal, whereas formulations showed to have pronounced cell-killing activity to HMWB cells in our study. All the three GBHs resulted in substantially decreased cell viability (< 23 %) from 500 μM (Roundup Mega and Glyfos) and from 750 μM (Fozat 480) concentrations. Exposure to GBHs also caused statistically significant ($p < 0.05$) increase of DNA damage from 500 μM (Roundup Mega and Glyfos) and 750 μM (Fozat 480). However, this observation cannot be explained by the direct genotoxic potential of GBHs, rather due to the high level of cytotoxic activity of the formulations, because cell death mechanisms, both apoptosis and necrosis, can produce spontaneous DNA fragmentation that can act as a confounder in the assessment of primary DNA damage in the comet assay. The high cytotoxic potential of GBHs may be attributed to the presence of other ingredients in the formulations, which has been reported in previous publications. POEA, the declared co-formulant in Glyfos, is more than 1000 times more cytotoxic than glyphosate alone, and concerns were also raised for its genotoxic potential at concentrations not causing cytotoxicity. Thus, EFSA concluded that POEA is clearly more toxic than glyphosate when tested in GBHs, and in response to this, Glyfos was withdraw from the Hungarian market in 2017. The ethoxylated etheralkylamine added to Roundup Mega has very similar toxicological properties as POEA, which is supported by the identical dose-response relationships of Roundup Mega and Glyfos, both of them containing surfactants at roughly the same concentration. The adjuvant content of Fozat 480 (< 5 % hygroscopic substances) could not be determined exactly from the MSDS; however, we can suspect from the path of the cell viability dose-response that it may also contain ethoxylated surfactants similarly to the other two GBHs. It is proven that ethoxylated adjuvants can be embedded into the cell membrane disrupting its integrity and permeability and therefore increasing the bioavailability of glyphosate. In this sense, surfactants in GBHs have an indirect synergistic effect with glyphosate. Researchers concluded that DNA damage observed in HMWB cells after exposure to glyphosate, Roundup 360 PLUS or the metabolite of glyphosate (aminomethylphosphonic acid, AMPA) was not due to direct interaction of these compounds with DNA as no DNA adducts formation has been observed, but rather ROS-mediated effects induced by the chemicals leading to cell death and indirect DNA damage. Previous studies have not been able to clearly demonstrate the oxidative DNA damaging potential of glyphosate; however, GBHs produced an increase in reactive oxygen species in both in vitro and in vivo test systems highlighting the role of

adjuvants in the cytotoxic effects observed in our experiments. Despite the definite cytotoxic effect, Roundup Mega and Fozat 480 are still commercially available not only in Hungary, but also in many other European countries under various brand names. In our study, the presence of metabolic activation did not alter the cytotoxic potential of either the active ingredient glyphosate or the GBHs. By contrast, we noted decreased DNA damages in the comet assay with Roundup Mega and Fozat 480, but increased damage with Glyfos as a result of S9 treatment. In humans, the only metabolite of glyphosate is the AMPA. It has been shown that AMPA is not able to induce DNA damage below a relatively high (4.5 mM) concentration, which is in agreement with our data. The diverse response of GBHs to metabolic activation observed in the comet assay may be explained by the presence of variable adjuvants in the formulations that underwent different metabolic modifications. Our results may indicate that the metabolite(s) of POEA in Glyfos can be more toxic than the parent compound in the comet assay, but not in the cell viability assay. There is no evidence in the published literature that POEA or other surfactants, or even products which contain these adjuvants, might undergo metabolic activation, and afterwards, become able to cause cytogenetic effects. The potential metabolic transformation of adjuvants can be hypothesized only from animal experiments; however, these studies have provided contradictory findings so far. This can still be considered as a critical knowledge gap to clarify the genotoxic potential of adjuvants in common commercial formulations of glyphosate under in vivo circumstances. A complex long-term experimental animal study has recently been initiated to assess possible risks resulting from the ubiquitous exposure to GBHs, and has already provided preliminary data on Roundup-induced endocrine effects and altered reproductive developmental parameters in male and female Sprague Dawley rats at a dose level considered as “safe”.

Conclusion

This is the first study that compared toxic effects of various glyphosate-based herbicide formulations to each other and with the declared active ingredient glyphosate in isolated human mononuclear white blood cells. GBHs caused much stronger cytotoxic effect on HMWB cells in comparison to glyphosate that may be attributed to the effect of various surfactants added to the formulations or their interaction with the active ingredient glyphosate and/or with other components of GBHs. Therefore, the GBHs-induced DNA damage observed in the comet assay could be most likely explained by non-genotoxic mechanisms and cannot indicate direct DNA damaging effects of glyphosate-based herbicide formulations. Nevertheless, by applying extended exposure durations and/or other test systems, such as the cytokinesis-block micronucleus cytome assay would allow for the discrimination between the cyto- and genotoxic effects and for the determination of the possible permanent genotoxic effect of these herbicides. Furthermore, this study, for the first time, pointed out the possibility that POEA containing formulation can undergo metabolic activation which draws attention to the need for comprehensive investigation of the toxicity of formulations to confirm our results and to assess the true health risks of environmental and occupational exposures.

Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes a well conducted comet assay using human lymphocytes as the test system to examine the genotoxicity and cytotoxicity of glyphosate and 3 commercial products containing glyphosate. No DNA damage was induced by analytical grade glyphosate. The 3 glyphosate products induced an increase in tail intensity in the comet assay only at highly cytotoxic concentrations, non-toxic concentrations induced no DNA damage.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Nagy *et al*., 2019	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		

Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity, content and storage conditions are not reported. Source: VWR International Kft, Debrecen, Hungary.
Only glyphosate acid or one of its salts is the tested substance	N	Also 3 GBH tested: Roundup Mega, Fozat 480, Glyfos.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Human mononuclear white blood cells.
Test conditions clearly and completely described	Y	Comet selection criteria were not stated. It is not stated if the slides were coded prior to scoring
Metabolic activation system clearly and completely described	Y	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	1, 10, 100, 250, 500, 750 and 1000 µM.
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	No HCD so it is unknown what degree of background variation is apparent in this test system. This is exacerbated by the use of a single set of control cultures
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and no positive control was used.		

Assessment and conclusion by RMS:

The publication examines the cytotoxicity and genotoxicity of glyphosate and three products containing glyphosate in an *in vitro* Comet assay using human mononuclear lymphocytes. Lymphocytes were treated for 4 h with 1-1000 µM glyphosate or with one of the three products at equivalent glyphosate concentrations. Exposure was with and without metabolic activation. Cytotoxicity was determined via fluorescent co-labelling and genotoxicity was determined by the alkaline comet assay.

Glyphosate did not induce significant cytotoxicity or genotoxicity in this study at any of the tested concentrations in the presence or absence of S9. The glyphosate-based products, in contrast, induced statistically significant cytotoxicity from 250 or 500 µM onwards. Statistically significant DNA damage (genotoxicity) was observed as well from 500 or 750 µM onwards. Metabolic activation significantly increased the DNA damage levels induced by one of the formulations, but not of the other formulations. The observed genotoxicity, however, may also be the result of severe cytotoxicity at those concentrations. Furthermore, cytotoxicity and genotoxicity of the formulations may also be attributed to co-formulants, especially considering that ethoxylated co-formulants are present in two of the three formulations.

No OECD GL exists which describes an *in vitro* comet assay. OECD GL 489 only deals with an *in vivo* comet assay; therefore procedures with regard to exposure and cell harvest cannot be considered to be conducted according to an OECD GL. However, the methods employed in this publication are similar to OECD GL 489 with regard to measuring genotoxicity, i.e. the comet assay itself. Nevertheless, some crucial information is missing and deviations from the guideline were noted (no HCD, results of positive control not shown, details about the test substance are missing, number of scored slides not in line with OECD GL 489). Based on these deviations, the study is considered supportive by the RMS.

B.6.4.4.4. *Genotoxicity – public literature, study 4*

Data point:	CA 5.4/004
Report author	De Almeida, L.K.S. *et al.*
Report year	2018
Report title	Moderate levels of glyphosate and its formulations vary in their cytotoxicity and genotoxicity in a whole blood model and in human cell lines with different estrogen receptor status
Document No	DOI: 10.1007/s13205-018-1464-z ISSN: 2190-572X
Guidelines followed in study	None (OECD GL 489 only describes *in vivo* comet assays, whereas an *in vitro* comet assay was performed within this publication; the comet assay itself, however, was conducted similarly to the procedure described in OECD GL 489)
Deviations from current test guideline	– Number of cells analysed in the genotoxicity test (50) not in line with OECD GL 489 (150 cells). – No information provided regarding metabolic activation. Considering that it is an *in vitro* assay, test conditions with and without metabolic activation should be investigated. – No HCD are available. Therefore, the proficiency of the research group cannot be proven. – Test substance characteristics of the formulations are not included in the publication.
Previous evaluation	Not applicable
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive

Full summary of the study according to OECD format

In vitro studies were conducted to determine the short-term cytotoxic and genotoxic effects of pure glyphosate and two glyphosate formulations (Roundup® and Wipeout®) at concentrations relevant to human exposure using whole blood (cytotoxicity) and various cancer cell lines (cytotoxicity and genotoxicity). Pure glyphosate (pure glyph) and Roundup® (Ro) showed similar non-monotonic toxicological profiles at low dose exposure (from 10 µg/ml), whereas Wipeout® (Wo) demonstrated a monotonic reduction in cell viability from a threshold concentration of 50 µg/ml, when tested in whole blood. We evaluated whether using various cancer cells (the estrogen-E2-responsive HEC1A, MCF7 and the estrogen-insensitive MDA-MB-231) exposed to moderate doses (75–500 µg/ml) would indicate varied toxicity and results indicated significant effects in the HEC1A cancer cells. A non-monotonic reduction in cell viability was observed in HEC1A exposed to pure glyph (75–500 µg/ml) and proliferative effects were observed after exposure to Wo (75, 125 and 250 µg/ml). Genotoxicity assessment (test concentration 500 µg/ml) demonstrated DNA damage in the HEC1A and MDA-MB-231 cells.

Materials and methods

Chemicals - Glyphosate (99.5 % purity) was purchased from Supelco Analytical (USA). Source of the formulations unknown.

Whole blood cell culture and exposure - Blood from 5 healthy volunteers was collected by venipuncture in heparin-containing tubes. Blood samples were diluted 1:10 in RPMI 1640 media supplemented with 50 µg/ml streptomycin and 50 U/ml penicillin. The 1 mL whole blood samples were exposed to glyphosate at various concentrations up to 500 µg/mL for 18 hours at 37 °C. Lipopolysaccharide (LPS, 5 µg/mL) was used as the positive control and pyrogen-free water as the negative control.

Breast cancer (MCF7 and MDA-MB-231) and endometrial cancer (HEC1A) cell line culture - MCF7 (hormone responsive) and MDA-MB-231 (hormone independent) cell lines were obtained from the American Type Culture Collection (ATCC), USA and HEC1A cells were obtained from Nelson Mandela Metropolitan University, Eastern Cape, South Africa. All cell lines were grown in DMEM supplemented with 5 % heat inactivated fetal calf serum, 50 U/mL penicillin and 50 µg/mL streptomycin. To maintain a stable estrogen-sensitive phenotype, cells were cultured in phenol-free medium after the removal of phenol red. All data were normalized against untreated controls. Cell lines were routinely maintained under standard cell culture conditions at 37 °C, 5 % CO2 and 90 % humidity. The different cell lines were exposed to glyphosate at varying concentrations up to 500 µg/mL for 24 hours at 37 °C. Camptothecin (100 µM), a DNA topoisomerase inhibitor, was used as the positive control in this study.

Cytotoxicity assay (MTT assay) - Following exposure to glyphosate, samples were incubated in 0.5 mg/mL MTT reagent for 3 hours for the cancer cell lines and for 30 minutes for whole blood at 37 °C. After incubation, the MTT reagent was aspirated and 1 mL of DMSO was added to solubilize the formazan product formed. Purple color formation was determined spectrophotometrically at 560 nm using a Biotek Powerwave XS microplate reader.

Single-cell gel electrophoresis (comet assay) - The exposure concentrations used for this study were chosen based on results obtained in the cell viability assay and the positive control reference concentration was chosen based on cytotoxicity results reported in the HepG2 human liver cell line. The MCF7, MDA-MB-231 and HEC1A cancer cell lines (100,000 cells/well) were incubated in 24-well plates for 4 hours at 37 °C, in the presence of 500 and 1000 µg/mL glyphosate. Camptothecin was used as a positive control in this study (100 µM). The preparation of the samples and the method used for the comet assay were conducted according to the instructions described in the OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc).

Preparation of cell samples for the comet assay - Trypsinized cells were pooled (three sample wells) and centrifuged at 700×g for 2 minutes. The supernatant was discarded and the pellet washed with ice cold PBS and centrifuged at 700×g. The cells (1×10^5 cells/mL) were then resuspended in ice cold PBS before the assay was conducted.

Comet assay sample slide preparation and cell lysis - Resuspended cells (10 µL) were combined with 100 µL molten comet agarose and the mixture (75 µL/well) was immediately placed onto an OxiSelect™ comet slide. Slides with the cell agarose mixture were incubated at 4 °C in the dark for 15 minutes to allow the agarose to set. Slides were treated in pre-chilled lysis buffer (pH 10) for 60 minutes at 4 °C in the dark followed by treatment in alkaline solution (pH 13) for 30 minutes at 4 °C in the dark.

Alkaline electrophoresis - Slides were subjected to alkaline electrophoresis for 18 minutes at 300 mA, neutralized in pre-chilled deionised water and washed in 70 % cold ethanol for 5 minutes. The slides were then air-dried and incubated with 100 µL of 1 × Vista Green dye prepared in TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) for 15 minutes at room temperature in the dark.

Comet assay sample visualization and data analysis - Slides were visualized with fluorescence microscopy (5×) using a FITC filter (7 %). Images were obtained using an AxioCam MR3 Camera and stored in the Axio vision Rel.4.8 program. Image J Macro was used to calculate the tail length, % tail moment and % tail DNA. 50 cells were analyzed per slide (replicates of 3) from pooled cell cultures, per experimental treatment (n = 3).

Statistical analysis - All data were presented as mean ± standard error of the mean (SEM). ANOVA single-factor analysis (Microsoft Excel) was used to determine significant differences ($P \leq 0.05$, $P \leq 0.01$). Non-linear least square regression models were applied to cytotoxicity data in human whole blood using the Graphpad Prism 6 software package. Goodness of fit was assessed using R2, R2 adjusted values and by the assessment of upper and lower 95 % confidence limits associated with the model fit. The Wald–Wolfowitz (runs) test was used to determine the deviation of the nonlinear regression model from the experimental data. The models were used to estimate the concentration of glyphosate required to illicit a half maximal response (half maximal effective concentration, EC50). Based on the observed biphasic nature of the curves obtained for glyphosate and Roundup, a model describing a seven-parameter bell-shaped dose (combines two sigmoidal responses)-response curve was selected.

Results

Cell viability in human whole blood – Glyphosate cytotoxicity in human whole blood at concentrations from 0.1 to 500 µg/mL was determined using the MTT reduction assay. A statistically significant reduction in cell viability of whole blood was observed for glyphosate at 10, 50 and 250 µg/mL but not at 500 µg/mL. LPS was a suitable positive control for this study.

Cell viability (MTT assay) in human cancer cell lines - A statistically significant reduction in cell viability was noted for glyphosate in the HEC1A cell line at 75, 125, 250 and 500 µg/mL. No change in viability was seen with the MCF7 and MDA-MB-231 cell lines.
The Roundup formulation did not induce significant cytotoxic effects on the three cancer cell lines at the concentrations tested. The Wipeout formulation showed no significant effects on the cell viability of the MCF-7 and MDA-MB-231 cell lines, however, a significant dose-independent increase in cell viability was observed in the HEC1A cell line at 75, 125, and 250 µg/ml.

The positive control, camptothecin, reduced significantly cell viability in all three cell lines when exposed to glyphosate, Roundup, or Wipeout.

Single cell gel electrophoresis (comet assay) - The test concentrations selected for genotoxicity studies were based on the results of the cell viability study and reference concentrations were chosen based on glyphosate concentrations reported in the literature to incur genotoxic damage in human cell lines. Glyphosate was tested in the comet assay at 500 and 1000 µg/mL where a statistically significant increase in tail length and tail moment was observed at both concentrations in the HEC1A and MDA-MB-231 cell lines, but not for the MCF7 cell line. The positive control, camptothecin, increased tail length significantly in the HEC1A and MDA-MB-231 cell lines but not in the MCF-7 cell line. The positive control increased tail moment significantly in all 3 cell lines.

Comet formation in the HEC1A, MCF-7 and MDA-MB-231 cell line after exposure to the glyphosate formulations Ro and Wo suggest that both Ro and Wo cause significant DNA damage in the HEC1A cell line at a concentration of 500 µg/mL and 800 µg/mL. Ro (500 µg/mL and 800 µg/mL) and Wo (500 µg/mL and 800 µg/ mL) did not stimulate significant comet formation in the MCF-7 cell line compared to the untreated control. The tail length results obtained for the MDA-MB-231 cell line indicates significant DNA damage by the Ro formulation at 500 µg/mL and 800 µg/ml. With respect to the Wo formulation, results indicated a significant increase in tail length at 500 µg/mL, but not at 800 µg/mL. Results for the 800 µg/mL dose, however, did not indicate a significant change in the tail length and tail moment for both Ro and Wo formulations. It is noted that the positive control camptothecin did not induce a significant increase in tail length of Roundup and Wipeout-treated HEC1A cell lines.

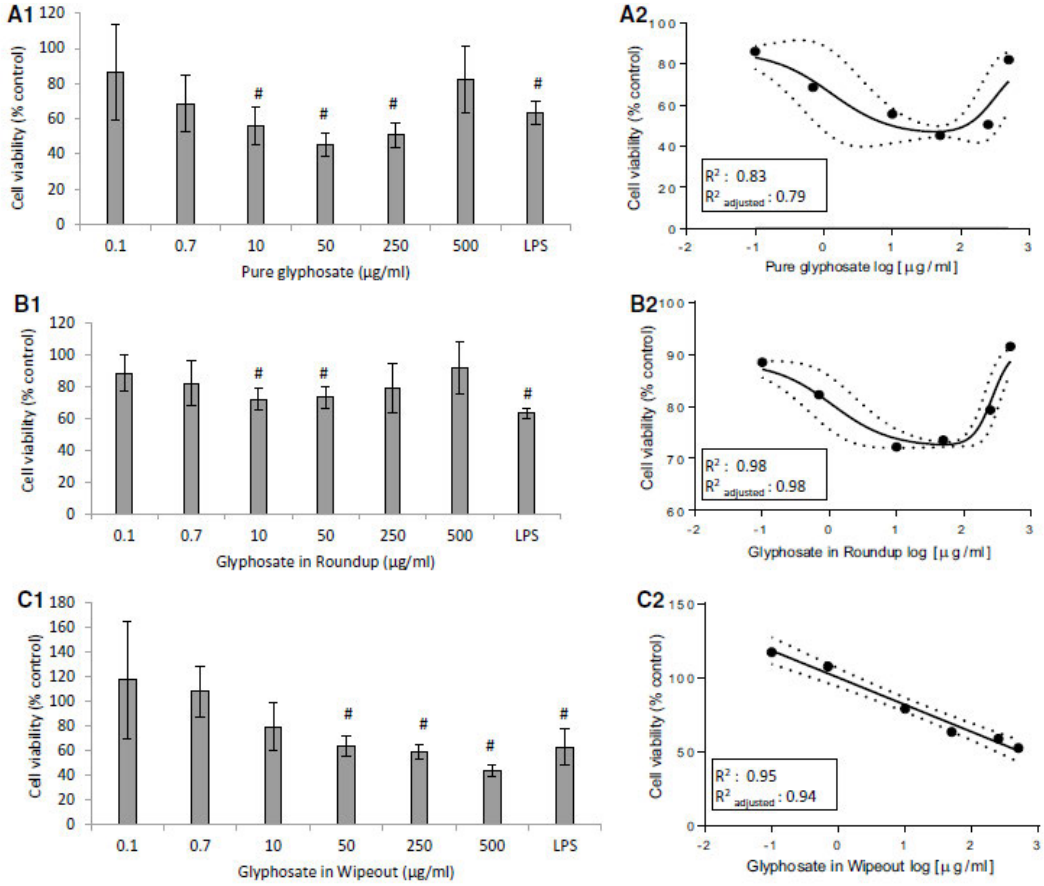


Fig. 1 The effect of **a1** pure glyph (99.5%) and its formulations, Ro (**b1**) and Wo (**c1**) on the cell viability in human whole blood. The cells were exposed over an 18-h period at varying herbicide concentrations (0–500 µg/ml). Lipolysaccharide (LPS, 5 µg/ml) was used as a positive control in this study. Data points represent the means of five replicates (±SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control (not shown) $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$. Non-linear regression analysis using Graphpad Prism 6 software was used to describe the effect of pure glyph (**a2**), Ro (**b2**) and Wo (**c2**) in human whole blood. **a2** Bell-shaped curve (biphasic curve): $Y\text{min} + (Y\text{max} - Y\text{min})/1 + 10^{(\log EC50_1 - \log[x]) * nH1} + Y\text{max}_2 - Y\text{min}/1 + 10^{(\log[x] - \log EC50_2) * nH2}$, **b2** bell-shaped curve (biphasic curve): $Y\text{min} + (Y\text{max} - Y\text{min})/1 + 10^{(\log EC50_1 - \log[x]) * nH1} + Y\text{max}_2 - Y\text{min}/1 + 10^{(\log[x] - \log EC50_2) * nH2}$, **c2** linear curve: $Y = m^{*}\log(x) + c$. Goodness of fit was assessed by R^2, R^2_{adjusted} values and upper and lower 95% confidence intervals around the fit (dash lines)

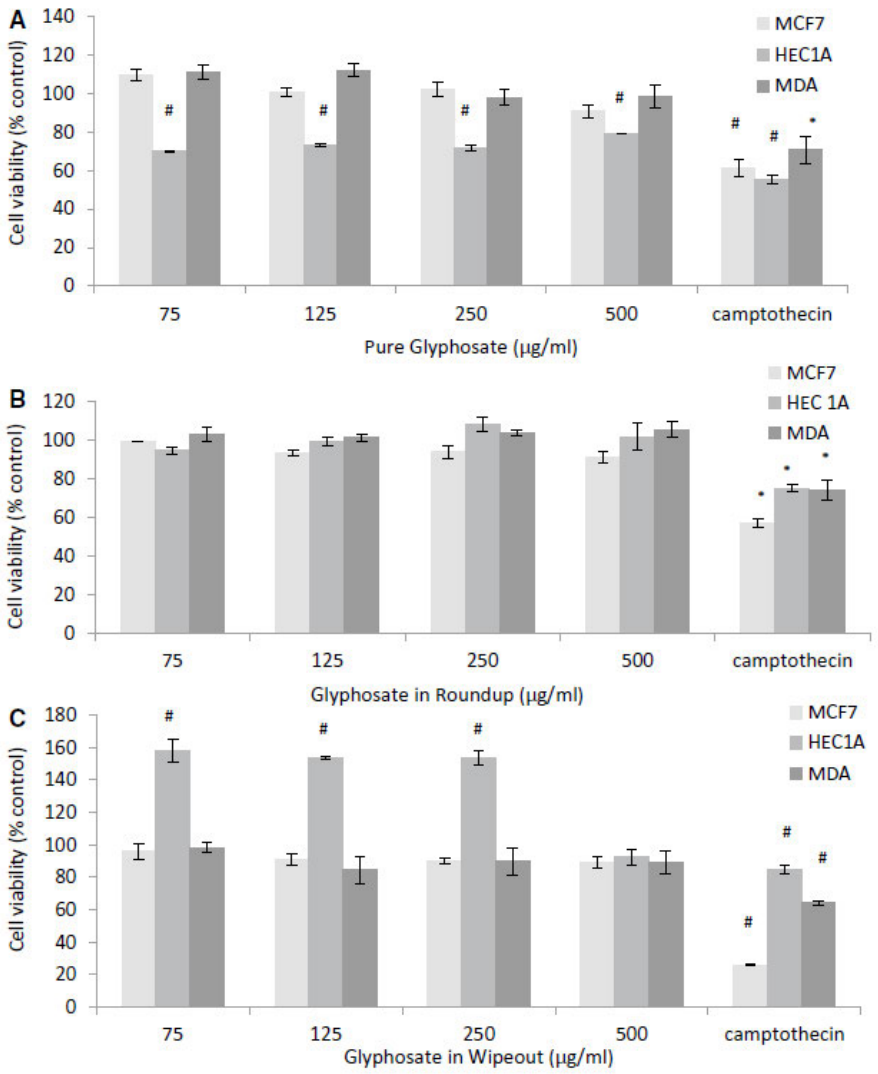


Fig. 2 The effect of **a** pure glyph (99.5%) and its formulations, Ro (**b**) Wo (**c**) on the cell viability of the cancer cell lines (MCF-7, HEC1A and MDA-MB-231). The cancer cell lines were exposed over a 24-h period at varying herbicide concentrations (0–500 µg/ml). Camptothecin (100 µM) was used as a positive control in this study. Data points represent the means of three replicates (±SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control (not shown) $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$

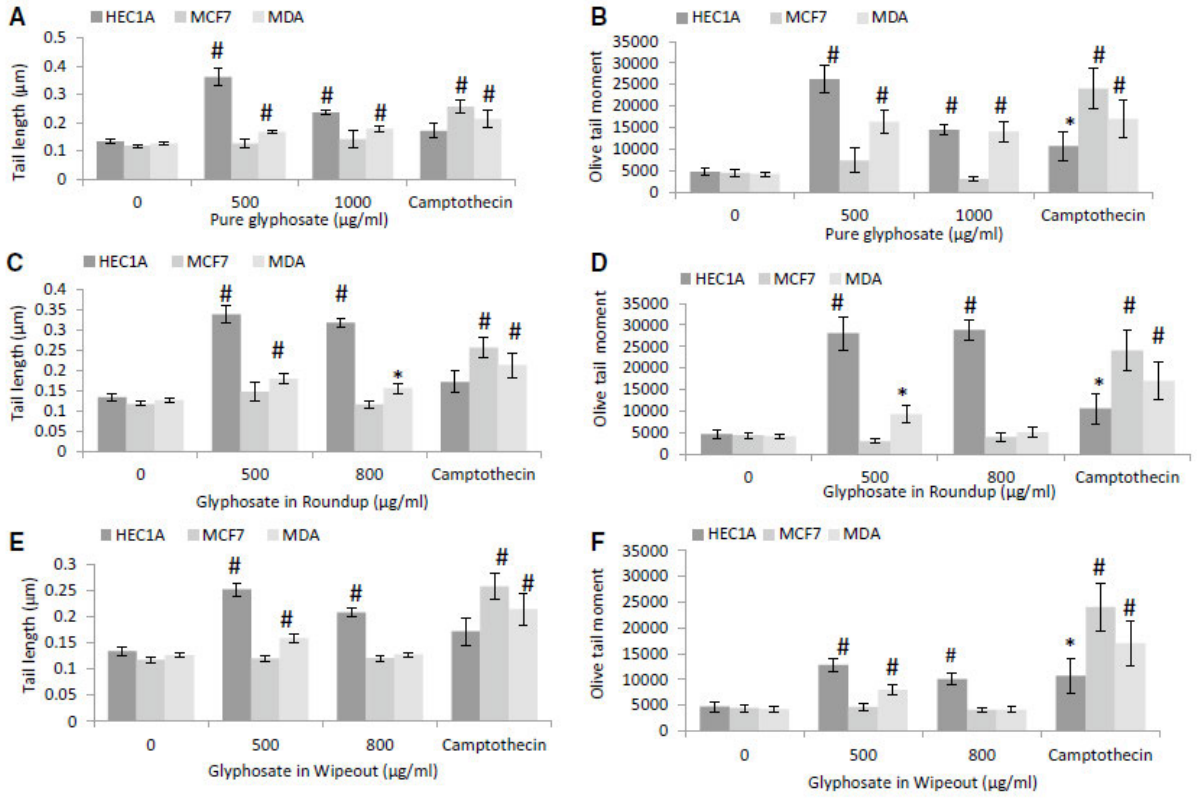


Fig. 4 DNA damage in HEC1A, MCF7 and MDA-MB-231 cancer cell lines presented as TL (µm): **a** pure glyph **c** Ro and **e** Wo and TM, **b** pure glyph, **d** Ro and **f** Wo. Cells were exposed for 4 h at 37 °C at test and reference herbicidal concentrations. Camptothecin (35 µg/ml) was the positive control in this study and data points represent the means of 50 cells analyzed per experimental treatment (± SEM). ANOVA single-factor analysis was used to determine significant differences from the untreated control. $^{*}P \leq 0.05$, $^{\#}P \leq 0.01$

Discussion and conclusions

When tested at concentrations ranging from 0.1 to 500 µg/mL, a statistically significant reduction in cell viability was observed in whole blood at glyphosate concentrations of 10, 50 and 250 µg/mL but not at 500 µg/mL. When tested for cytotoxicity glyphosate showed a statistically significant reduction in cell viability in the endometrial cancer cell line HEC1A at 75, 125, 250 and 500 µg/mL. No effect on cell viability was seen on hormone responsive (MCF7) and hormone independent (MDA-MB-231) breast cancer cell lines at concentrations up to 500 µg/mL glyphosate. When glyphosate was tested for DNA damage in the single cell gel electrophoresis assay (comet assay) a statistically significant increase in tail length and tail moment was observed at 500 and 1000 µg/mL in the endometrial cancer cell line HEC1A and the hormone independent breast cancer cell line MDA-MB-231. No DNA damage was observed in the hormone responsive breast cancer cell line MCF7 up to concentrations of 1000 µg/mL. Cytotoxicity results at concentrations relevant to occupational and residential exposure to glyphosate observed in the three cancer cell lines suggest that toxicity varies depending on cell type, with the most significant results observed in the HEC1A cancer cell line exposed to glyphosate. Moderate concentrations of glyphosate (500 µg/mL) induced genotoxic effects in the HEC1A and MDA-MB-231 cancer cell lines, which suggests that glyphosate may display various mechanisms of toxicity.

Assessment and conclusion

Assessment and conclusion by applicant:

The cytotoxicity of glyphosate has been investigated in whole blood, in hormone independent (MDA-MB-231) and in hormone responsive (MCF7) cell lines and in a an endometrial cancer cell line (HEC1A). The capacity of glyphosate to produce DNA damage was investigated in MCF7, MDA-MB-231 and HEC1A cells in the Comet assay. Glyphosate was found to reduce cell viability in whole blood at the intermediate

concentrations (10-250 µg/mL) but not at the highest concentration tested (500 µg/L). A significant concentration related reduction in cell viability was seen with glyphosate in HEC1A cells (> 75 µg/mL) but not in the two other cell lines. When glyphosate was tested at 500 and 1000 µg/mL an increase in tail length and tail moment was observed in HEC1A and MDA-MB-231cells but not in the hormone responsive breast cancer cell line MCF7. The *in vitro* concentrations of glyphosate at which DNA damage was observed were 500 and 1,000 µg/mL which are systemic concentrations that cannot be reached in *in vivo* toxicology studies.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the Comet assay was only conducted at concentrations that are physiologically not feasible in *in vivo* toxicology studies (> 1mM).

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: De Almeida et al., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 99.5 %. Source: Supelco Analytical USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also glyphosate-based formulations were tested.
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Whole blood from volunteers, breast cancer cells (MCF7 and MDA‑MB‑231) and endometrial cancer cells (HEC1A).
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	N	For cytotoxicity testing glyphosate concentrations from 0.1 to 500 µg/mL were used. For comet testing only glyphosate concentrations of 500 and 1000 µg/mL were used (> 1 mM).
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Was studied but not established.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the Comet assay was only conducted at concentrations that are physiologically not feasible in *in vivo* toxicology studies (> 1mM).		

Assessment and conclusion by RMS:

It is agreed with the applicant's summary and conclusions of the journal article. It is noted, however, that the applicant did not summarise results regarding the tested formulations Roundup and Wipeout, but only results regarding the active substance itself. For completeness, the RMS included some information about the formulations as well in the results section of the study summary. In addition, no figures were included by the applicant either. For the sake of completeness, the RMS included some screenshots of the results as shown in the journal article.

Glyphosate was found to reduce cell viability in whole blood at the intermediate concentrations (10-250 µg/mL) but not at the highest concentration tested (500 µg/L). No clear explanation is given for this phenomenon. The same pattern was noted for Roundup. Cytotoxicity was also seen for Wipeout, in this case with a dose-response relationship. Cell viability of HEC1A cells was decreased in a dose-dependent manner at glyphosate concentrations of (> 75 µg/mL) but not in the two other cell lines. For Roundup, no cytotoxicity was seen in any of the three cell lines. And for Wipeout a significant decrease in cell viability was observed only in the HEC1A cell line, but not in the other two cell lines.

The *in vitro* comet assay was positive HEC1A cell line and negative in the MCF7 cell line for glyphosate and both formulations when tested at 500 and 800/1000 µg/mL. In the MDA-MB-231 cell line a positive result was observed for glyphosate and for Roundup except at 800 µg/mL with regard to tail moment and for Wipeout at 500 µg/mL, but not at 800 µg/mL. It is noted that the *in vitro* concentrations of glyphosate at which DNA damage was observed were 500 and 800/1,000 µg/mL which are systemic concentrations that cannot be reached in *in vivo* toxicology studies.

The RMS notes that there are only two high concentrations assessed in this *in vitro* Comet assay, about 3-6 mM. These are very high concentrations, which do not seem to occur *in vivo*, and a wider range of concentrations is missing. Furthermore, at the highest concentration (800 and 1000 µg/ml) the cytotoxicity has not been assessed. It is, thus, difficult to interpret this study since the cytogenicity assay does not indicate any dose-response relationship.

No OECD GD exists which describes an *in vitro* comet assay. OECD GD 489 only deals with an *in vivo* comet assay; therefore procedures with regard to exposure and cell harvest cannot be considered to be conducted according to an OECD GD. However, the methods employed in this publication are similar to OECD GD 489 with regard to measuring genotoxicity, i.e. the comet assay itself. Nevertheless, some crucial information is missing and deviations from the guideline were noted (details regarding the tested formulations missing, no HCD, no information regarding metabolic activation, number of scored slides not in line with OECD GL 489).

Based on these deviations, the study is considered supportive by the RMS.

B.6.4.4.5. *Genotoxicity – public literature, study 5*

Data point:	CA 5.4/005
Report author	Ilyushina, N.A. *et al.*
Report year	2018b
Report title	Comparative investigation of genotoxic activities of glyphosate technical products in the micronucleus test in vivo
Document No	ISSN: 0869-7922
Guidelines followed in study	OECD Test Guideline 474
Deviations from current test guideline OECD 474 (2016)	Positive control animals were included but the data are not reported. No ratio of PCE to NCE was reported. No evidence of bone marrow exposure. Data have been presented per group rather than per animal. HCD data not included.
Previous evaluation	None
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is not considered reliable due to the deviations noted above.

Full summary of the study according to OECD format
In this study, the induction of micronucleus formation in vivo in polychromatophilic erythrocytes of bone marrow of CD-1 mice was assessed by the action of three different technical glyphosate products on the market in the Russian Federation. It was found that the tested samples of technical products showed different cytogenetic activity, while only one of them caused a statistically significant, dose-dependent increase in the frequency of induction of micronuclei compared to the negative control.

The analysis of the composition of the studied product samples showed that the cytogenetic activity may depend on the content of potentially mutagenic impurities, formaldehyde in particular.

The acquired data provides some additional grounds for lowering the upper limit of formaldehyde content in technical glyphosate products and also indicates the need to assess the genotoxic activity of analogue pesticides entering the market of plant protection products.

Materials and methods
Three glyphosate technical products were used that were manufactured at different factories and that contained the active substance (glyphosate acid) in amounts of 96.6 %, 95.8 % and 95.7 % in technical products I, II and III, respectively.

Chromosomal disturbances were revealed through the use of in vivo micronucleus analysis [OECD Test No. 474: Mammalian Erythrocyte Micronucleus Test. 2016] of the bone marrow erythrocytes in CD-1 laboratory mice obtained from the husbandry center at the Andreyevka Branch of the Federal State Budget Research Institution "Biomedical Technology Science Center" of the Russian Federal Medicobiological Agency.

Five mice per group were used. Technical glyphosate was administered intragastrically in 1 % starch in doses of 500, 1,000 and 2,000 mg/kg of body weight (the maximum dose according to OECD Test No. 474) twice, twenty-four hours apart, and the animals were euthanized 22 hours following the second administration. At the same time, an excipient (1 % starch) was given as a negative control following the same pattern as was used for the technical glyphosate. Cyclophosphamide was used as a positive control, given intragastrically in the amount of 40 mg/kg once, at the same time as the second dose of glyphosate.
Bone marrow preparations from each specimen were studied microscopically (on a Japan-made Nikon Eclipse Ci-L), counting 4,000 polychromatophilic erythrocytes (PCEs) for measuring micronucleus frequency), and the portion among the erythrocytes as a whole made up by PCEs, counting no fewer than 500 erythrocytes for each animal.

A statistical analysis of the study results was carried out using SPSS Statistics v. 22.0 (IBM, New York, USA). Frequencies of micronuclei in the PCEs were compared by building a generalized log-linear model of the Poisson Regression ($\alpha = 0.05$). The data were also checked for dependencies between micronucleus frequency and the dose of the technical product being tested using the Mantel-Haenszel method.

Results
All the research was carried out in accordance with OECD Manual No. 474 and Methodological Instructions MU-1.2.3365-16. Each experiment used positive and negative controls simultaneously. The frequency of PCEs containing micronuclei in the case of the positive control was in a range from 1.58 to 2.95 %, while in the case of the negative control it was from 0.06 to 0.12 %. All the values were within the range of the distribution boundaries of historical laboratory controls.

The quantification of the portion of total erythrocytes made up by polychromatophilic erythrocytes show that in none of the analyses was erythropoiesis suppressed, i.e. there were no toxic effects on mouse bone marrow.

Varying effects were revealed during the study of the mutagenic activity of the glyphosate technical products through micronucleus analysis. Technical products II and III did not significantly increase the PCE frequency in mouse bone marrow compared to the simultaneous negative controls (please refer to Table and Figure below).

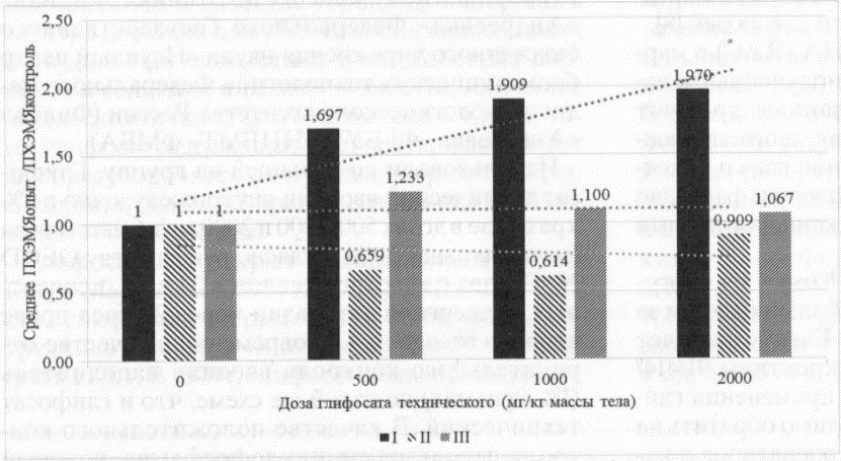


Figure B.6.4.4.5-1: Technical Glyphosate Dose (mg/kg of body weight) Frequencies of increased occurrence of PCEs compared to the negative control depending on the dose of glyphosate technical products. I, II, III – Reference numbers for different technical products. $PCEMN_{exp}$ denotes the frequency of PCEs with micronuclei in groups of mice that received different glyphosate doses. $PCEMN_{control}$ denotes the frequency of PCEs with micronuclei in groups of mice that received an excipient with 1 % starch (negative control) (from: Ilyushina *et al*., 2018)

When administering technical Product I, a low, but statistically significant genotoxic effect was observed at all doses (Table 2). Moreover, a statistically significant linear dependency was observed of the frequency of PCEs with micronuclei upon the dose of the compounds under study (p= 0.002) (please refer to the Table below).

A comparison was made between the content of ingredients in the study samples based on the available batch analysis certificates for the technical products. Two ingredients were of special interest from the point of view of genotoxicity: nitroso-glyphosate and formaldehyde. According to the provided certificates, nitrosoglyphosate was not present in any of the batches of technical products (its content, in accordance with FAO specifications, must not exceed 1 mg/kg). Formaldehyde content in products I, II and III amounted to 0.13 %, 0.024 % and 0.06 %, respectively. A formaldehyde level in the glyphosate product of 0.13% is the maximum permissible concentration in glyphosate technical products in accordance with the FAO specifications. However, it should be noted that, according to Annex I of Directive 91/414/EEC, as amended in 2017, the partial content of formaldehyde in glyphosate must not exceed 1 mg/kg.

A comparison of the results we have obtained on the mutagenic activity and formaldehyde concentrations in glyphosate technical samples showed that statistically significant genotoxic effects and a linear dependence of the frequency of PCEs with micronuclei upon the dose of the compound under study were only observed in the case of the product with 0.13% formaldehyde. Lower concentrations of this ingredient did not induce the statistically significant formation of PCEs with micronuclei in bone marrow cells.

Thus, the observed mutagenic effects were most likely not related to the active ingredient, but rather to the formaldehyde contained as an ingredient in the products.

Table B.6.4.4.5-1: Probable profile (95%) of confidence intervals for the frequency of polychromatophilic erythrocytes with micronuclei (MN) under the effects of different doses of glyphosate technical products relative to the negative control

Glyphosate dose (mg/kg of body weight)	Sample I		Sample II		Sample III	
	Number of PCEs with MN per 40,000 PCEs	95 % confidence intervals (lower limit, mean, upper limit)	Number of PCEs with MN per 40,000 PCEs	95 % confidence intervals (lower limit, mean, upper limit)	Number of PCEs with MN per 40,000 PCEs	95 % confidence intervals (lower limit, mean, upper limit)
0	33	1	44	1	30	1
500	56	1.111 1.697 2.634	29	0.408 0.659 1.048	37	0.763 1.233 2.009
1000	63	1.263 1.909 2.941	27	0.376 0.614 0.984	33	0.671 1.100 1.812
2000	65	1.306 1.970 3.028	40	0.591 0.909 1.395	32	0.647 1.067 1.762
Assessment of linear trend using the Mantel-Haenszel method	1 (146)		II (189)		III (341)	
	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)	Value	Asymptomatic significance p (one-dimensional)
	9.791	0.002	0.28	0.597	0.06	0.938

The results obtained are in agreement with data from the literature. In the majority of studies conducted using the Ames test, glyphosate did not induce gene mutations in bacteria [Li A.P., Long T.J., 1988]. In both *in vivo* and *in vitro* micronucleus analyses carried out on different subjects, contradictory data were obtained. An increased frequency of the appearance of micronuclei was noted in several studies on cultivated human buccal epithelial cells [Koller V.J. et al, 2012], in mouse bone marrow [Manas F., 2009], and in human lymphocytes having undergone been affected by glyphosate-containing preparations during crop spraying [Bolognesi C. eta la, 2009]. However, the majority of the studies gave negative results in terms of micronucleus analyses [Kier L.D., 2013]. However, none of the studies analyzed the content of possible mutagenic ingredients in technical products and glyphosate-containing preparations and their contribution to the observed effects.

Conclusion

The comparative investigation of the genotoxic activity of various glyphosate technical products through the analysis of micronuclei in mouse bone marrow erythrocytes showed that cytogenetic activity may depend on the content in the product of potentially mutagenic ingredients, and in particular of formaldehyde. The contradictory results obtained in different studies of the mutagenic activity of glyphosate and preparations containing it may be connected to the different concentrations of ingredients that may exhibit effects of intracellular genetic structures.

The obtained data comprise additional substantiation for reducing the upper limit for formaldehyde content in glyphosate technical products, and also provide evidence of the need to assess the genotoxic activity of analogous pesticides entering the Russian Federation's market.

Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes the results of three different technical batches of glyphosate tested in a mouse micronucleus test. The study followings the recommendations of OECD 474, with some deficiencies, mostly regarding the reporting of data rather than test methodology. However, the reliability of the reported conclusions are unknown due to a lack of clarity and accuracy of the reported data. Individual animal data and group mean±SD frequency of micronucleated (MN) PCE have not been reported. Instead the results per group

appear to be presented as the total number of MN PCE found per 40,000 PCE, together with the frequency of MN PCE values, expressed as relative to control, for the group with calculated 95 % upper and lower limits (no explanation for how these data were derived is provided). The text describes the vehicle control MN PCE frequencies as ranging between 0.06 % and 0.12 % (within HCD) but similar detail is not provided for the treated groups. Consequently, there is no indication of animal variability within the groups and it is unknown if any of the treated animals fall outside of HCD. Furthermore, the total number of MN PCE is described as being per 40,000 PCE, however, only 4000 PCE were scored per animal and with 5 animals per group this would result in total of 20,000 PCE per group.

The authors postulate that the positive results observed for technical batch I are likely to be due to the presence of 0.13% formaldehyde in the material, although they provide no data to support their hypothesis.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions and the data are inadequately reported.

Reliability criteria for *in vivo* toxicology studies made by the applicant

Publication: Ilyushina *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	OECD test guideline 474 according to the authors.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Technical glyphosate from 3 sources with purity of 96.6 %, 95.8 %, and 95.7 %. Sources were not reported but the concentration of impurities (nitroso-glyphosate and formaldehyde) was given.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	CD-1 mouse.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0, 500, 1,000, and 2,000 mg/kg bw twice, twenty-four hours apart.
Positive control	Y	Cyclophosphamide.
Number of animals used per dose level reported	Y	5/dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	Y	Referred to but not presented.
Dose-effect relationship reported	Y	

Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the source of the 3 glyphosate batches was not revealed although the concentrations of the most important impurities were given for each batch. Although reference was made to OECD test guideline 474 too little detail was given on the conduct of the MN assay to conclude to reliability without restrictions, and the data are inadequately reported.		

Assessment and conclusion by RMS:
It is agreed with the assessment by the applicant as described above.
It is noted that a positive result for batch I was postulated as due to the presence of 0.13% formaldehyde as an impurity. However, no data is submitted to support this hypothesis. The RMS notes that no evidence for induced systemic mutations is available for formaldehyde (CLH opinion on formaldehyde, 2012) and it is questionable whether 0.13% of an impurity would induce micronuclei *in vivo*.

In contrast to the applicant, however, the RMS does not consider the study to be reliable with restrictions. Due to the noted deviations (positive control animals were included but the data are not reported. No ratio of PCE to NCE was reported. No evidence of bone marrow exposure. Data have been presented per group rather than per animal. HCD data not included) from the OECD guideline and the inadequately reported results in the publication, the study is considered supportive.

B.6.4.4.6. *Genotoxicity – public literature, study 6*

Data point:	CA 5.4/006
Report author	Santovito, A. *et al.*
Report year	2018
Report title	In vitro evaluation of genomic damage induced by glyphosate on human lymphocytes
Document No	doi.org/10.1007/s11356-018-3417-9 E-ISSN: 1614-7499
Guidelines followed in study	Some compliance with OECD 473 and OECD 487

Deviations from current test guideline	– Only continuous treatments in the absence of S9 were performed and these exceeded the 1.5 cell cycles recommended by both guidelines. Considering that it is an *in vitro* assay, test conditions with and without metabolic activation should have been investigated. – No historical control data are reported; proficiency of the lab not demonstrated. – Highest dose (0.500 µg/mL) not in line with OECD guidelines (2000 µg/mL). Since no cytotoxicity was observed, higher concentrations should have been tested. – Treatment with glyphosate was initiated 24 hours after lymphocyte cultures were stimulated to divide, instead of the recommended 48 hours. – Exposure to glyphosate was continuous for 28 hours in the chromosome aberration assay or 48 hours in the micronucleus assay. In contrast OECD test guidelines recommend maximum exposure of 1.5 cell cycles, equivalent to approximately 24 hours for lymphocyte cultures. – No data provided on stability of the test substance and the tested concentration not analytically verified.
Previous evaluation	No
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive due to the deviations noted above.

Full summary of the study according to OECD format

In this study, the *in vitro* clastogenic and/or aneugenic effects of glyphosate was analysed by chromosomal aberrations and micronucleus assays. Human lymphocytes were exposed to five glyphosate concentrations: 0.500, 0.100, 0.050, 0.025, and 0.0125 µg/mL, where 0.500 µg/mL represents the established acceptable daily intake value, and the other concentrations were tested in order to establish the genotoxicity threshold for this compound.
The study authors observed that chromosomal aberration (CA) and micronuclei (MNi) frequencies significantly increased at all tested concentrations, with exception of 0.0125 µg/mL. Vice versa, no effect has been observed on the frequencies of nuclear buds and nucleoplasmic bridges, with the only exception of 0.500 µg/mL of glyphosate that was found to increase in a significant manner the frequency of nucleoplasmic bridges. Finally, the cytokinesis-block proliferation index and the mitotic index were not significantly reduced, indicating that glyphosate does not produce effects on the proliferation/mitotic index at the tested concentrations.

Materials and Methods
Study population; Peripheral venous blood was collected from six healthy Italian subjects (two males and four females, mean age ± SD, 27.50 ± 12.55), non-smoking, not alcoholics, not under drug therapy, and with no recent history of exposure to mutagens. All subjects signed the informed consent. The study was approved by the University of Turin ethics committee and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki.

Blood sample collection and lymphocyte cultures; Blood samples were obtained by venipuncture, collected in heparinized tubes, cooled (4 °C), and processed within 2 h after collection. Lymphocyte cultures, fixation, and staining procedures were performed as previously described. Total time of lymphocyte cultures was 52 and 72 h for CA and MNi assays, respectively.
After 24 h of incubation, 8.6 µL of glyphosate stock solution (Sigma-Aldrich, Saint Louis, MO, USA, CAS n. 1071-83-6) at the final concentration of 0.5 mg/mL in dimethyl sulfoxide (DMSO) was added to the lymphocyte culture in order to reach a final glyphosate concentration of 0.500 µg/mL. Similarly, 8.6 µL of glyphosate stock solution diluted 5, 10, 20, and 40 times with DMSO were added to the lymphocyte cultures in order to reach the final glyphosate concentrations of 0.100 µg/mL, 0.050 µg/mL, 0.025 µg/mL, and 0.0125 µg/mL, respectively. In particular, 0.500 µg/mL represents the ADI concentration established by EFSA for this compound, whereas 0.100, 0.050, 0.025, and 0.0125 µg/mL concentrations were tested in order to evaluate the genotoxicity threshold.
Three control cultures were assessed: (1) positive control, by adding only MMC (final concentration 0.1 µg/mL culture); (2) 0.1 % DMSO solvent control, obtained by adding 8.6 µL of DMSO to the lymphocyte culture; and (3) negative control culture without both glyphosate and DMSO, obtained adding 8.6 µL of RPMI medium to the lymphocyte culture.
Only for MNi assay, after 44 h of incubation, cytochalasin-B was added to the cultures at a concentration of 6 µg/mL to block cytokinesis. Similarly, only for CA assay, to arrest cells in mitosis, colchicine was added at the concentration of 0.06 µg/mL during the last 2 h of culture.
After 52 h (for CAs assay) and 72 h (for MNi assay) of incubation at 37°C, the cells were collected by centrifugation, treated for 10 min with a pre-warmed hypotonic solution (75 mM KCl). After centrifugation and removal of the supernatant, the cells were fixed with a solution of methanol/acetic acid (3:1 v/v). The treatment with the fixative was repeated three times. Finally, the supernatant was discarded, and the pellet, dissolved in a minimal volume of fixative, was seeded on the slides to detect CAs and MNi by conventional staining with 5 % Giemsa (pH 6.8) prepared in Sörensen buffer.

Cytokinesis-block micronucleus assays; Microscope analysis was performed at ×400 magnification on a light microscope (Dialux 20, Leica, Germany). MNi, nucleoplasmic bridges (NPB), and nuclear buds (NBUD) were scored in 2000 binucleated lymphocytes with well-preserved cytoplasm per subject (total 12,000 binucleated cells per concentration). Cells containing one of more MNi were scored as "micronucleated cell" (MNC). A total of 2000 lymphocytes per donor per concentration were scored to evaluate the cytokinesis-block proliferation index (CBPI), according to the following formula: [1 × N1] + [2 × N2] + [3 × (N3 + N4)] / N, where N1–N4 represents the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored.

Chromosomal aberration assay; Microscope analysis was performed at ×1000 magnification on a light microscope (Dialux 20, Leica, Germany). For each subject and glyphosate concentration, 200 complete complete metaphases (for a total of 1200 metaphases for each dose) were analyzed. Cells containing one of more types of CAs were scored as "aberrant cell" (Ab.C). In order to determine cytotoxicity, the mitotic index (MI) was calculated from the number of metaphases in 1000 cells analyzed per subject per concentration (a total of 6000 cells per concentration).

Statistical analysis; Comparison of mean values of the percentage of cells with MNi, MNC, CBPI, NPBs, NBUDs, CAs, Ab.C, and MI between exposure levels and controls was assessed by the nonparametric Mann-Whitney test. Statistical calculations were carried out using the SPSS software package program (version 24.0, Inc., Chicago, IL, USA). All P values were two-tailed, and P values of 5 % or less were considered statistically significant for all tests carried out.

Results

Effect of glyphosate on CA formulation; Table B.6.4.4.6-1 shows values of CAs found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate was found to induce the following structural CAs: gaps, chromatid and chromosome breaks, dicentric chromosomes, rings, tri-tetraradials, and acentric fragments. This last, together to chromatid breaks, represent the most frequent observed aberrations (Table below). Gaps were excluded from statistical analysis. Glyphosate was found to significantly increase the CA and Ab.C frequencies at all tested concentrations when compared with the solvent control, including the concentration of 0.025 μg/mL, but with the exception of 0.0125 μg/mL. A dose effect was also observed, since the regression analysis revealed a significant correlation between glyphosate concentrations and the CA and Ab.C frequencies. Vice versa, no significant differences were found between the DMSO solvent-control and the negative control, whereas the cultures treated with the MMC showed a significant increase of the cytogenetic damage with respect to all concentrations of glyphosate. Finally, no significant differences were found in the MI values between solvent control and all tested concentrations of glyphosate, although at 0.500 μg/mL, the P value resulted to be borderline.

Table B.6.4.4.6-1: Induction of chromosomal aberrations by Glyphosate in human lymphocytes *in vitro* Number if scored metaphases for each concentration = 1200 (from: Santovino *et al*., 2018)

Test substance (µg/mL)	Structural CAs							Total CAs	Total CAs + Gaps	Total Ab.C	Total Ab.C + Gaps	(%) CAs/Cell ± S.E.	(%) Ab.C/Cell ± S.E.	(%) MI ± S.E.
	Gaps	B'	B"	DC	R	TR	AF							
NC	8	8	2	0	0	0	7	17	25	17	25	1.417±0.154	1.417±0.154	5.567±0.042
0.1% DMSO	10	9	4	1	0	6	7	27	37	27	37	2.250±0.335	2.250±0.250	5.433±0.056
MMC (0.100)	41	46	30	9	10	14	36	145	186	127	168	12.083±0.300[a]	10.583±0.473	4.200±0.058[a]
Gly (0.500)	17	41	12	12	3	0	28	96	113	95	112	8.000±0.428[a]	7.917±0.375	5.300±0.026
Gly (0.100)	23	31	10	7	2	2	23	75	98	75	98	6.250±0.359[a]	6.250±0.359	5.333±0.080
Gly (0.050)	9	21	6	7	0	0	16	50	59	50	59	4.167±0.167[a]	4.167±0.167	5.367±0.095
Gly (0.025)	10	15	4	4	3	0	20	46	56	46	56	3.833±0.211[b]	3.833±0.211[b]	5.383±0.040
Gly (0.0125)	8	14	5	1	0	0	14	34	42	34	42	2.833±0.211	2.833±0.211	5.400±0.037

CAs, chromosomal aberrations; *Ab.C*, aberrant cells (cells with 1 ore more aberrations); *MI*, Mitotic Index; *NC*, Negative Control; *MMC*, Mitomycin-C; *B'*, chromatid break; *B"*, chromosome break; *DC*, dicentric; *R*, ring; *TR*, tri-tetraradials; *AF*, acentric fragments; *S.E.*, standard error; *Gly*, Gliphosate

[a] $P = 0.004$; [b] $P < 0.006$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Table B.6.4.4.6-2: Multiple regression analysis between Glyphosate concentrations (from: Santovino *et al*., 2018).

		(Lower) – (Upper)	
CAs	0.914	(2.112) – (2.988)	<0.001
Cells with CAs	0.919	(2.099) – (2.935)	<0.001
MI	-0.275	(-0.666) – (0.099)	0.141
MNi	0.908	(4.025) – (5.075)	<0.001
Cells with MNi	0.935	(3.639) – (4.527)	<0.001
CBPI	0.269	(-28.171) – (4.571)	0.151
NPBs	0.674	(0.268) – (0.665)	<0.001
NBUDs	0.395	(0.023) – (0.444)	0.031

CAs, Chromosomal Aberrations; *MI*, Mitotic Index; *MNi*, Micronuclei; *CBPI*, Cytokinesis-block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds

Effect of glyphosate on MNi formulation; Table B.6.4.4.6-3 shows the frequencies of MNi found in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Glyphosate significantly increased the MNi frequency at all tested concentrations when compared to the solvent control, with exception of 0.0125 μg/mL (Table B.6.4.4.6-3). Vice versa, no effect has been observed on the frequencies of NBUD and NPB, with the only exception of 0.500 μg/mL of glyphosate that was found to increase in a significant manner the frequency of NPBs with respect to the solvent control. Also in this case, a relationship between the frequency of MNi and the concentrations of glyphosate was observed (Table B.6.4.4.6-2), as well as the DMSO solvent-control cultures did not show significant differences with respect to the negative controls. MMC was found to significantly increase the MNi, NPB, and NBUD formation compared with the negative control solvent controls and all tested concentrations of glyphosate, with exception of 0.500 μg/mL. After 48-h exposure, a significant reduction of the CBPI value in cultures treated with glyphosate was not observed, indicating that, at the tested concentrations, glyphosate does not seem to produce effects on the proliferation index. Finally, at 0.500 μg/mL, glyphosate significantly induced the NPB formation, whereas no differences were found in the frequency of NBUD between DMSO solvent control and all glyphosate concentrations.

The RMS notes that nucleoplasmic bridges and nuclear buds are markers for cytotoxicity/cytostasis, but according to OECD guideline 487 these may be determined in studies without the use of a cytokinesis blocker (CytoB in case of the current publication). Therefore, the relevance of these markers in the frame of the current journal article is questioned.

Table B.6.4.4.6-3: Induction of micronuclei by Glyphosate in human lymphocytes in vitro. Number of scored binucleated cells for each concentration of the test substance = 12,000 (from: Santovino *et a*l., 2018)

Test substance (μg/mL)	Distribution of BNCs according to the number of MNi				MNi	MNC	MNi/BNCs ± S.E. (%)	MNC/BNCs ± S.E. (%)	CBPI ± S.E	Frequency of BNCs with NPBs (‰)	Frequency of BNCs with NBUDs (‰)
	1	2	3	4							
NC	27	0	0	0	27	27	0.225±0.021	0.225±0.021	1.713±0.003	0.417±0.083	0.833±0.105
0.1% DMSO	33	1	0	0	35	34	0.292±0.024	0.283±0.025	1.589±0.076	0.500±0.129	1.083±0.154
MMC (0.100)	129	9	3	2	164	143	1.367±0.067	1.192±0.015[a]	1.366±0.019	2.333±0.357	3.083±0.473 [a]
Gly (0.500)	132	6	2	1	154	141	1.283±0.017	1.175±0.021[a]	1.545±0.054	1.667±0.211	1.666±0.247
Gly (0.100)	107	7	0	0	121	114	1.008±0.030	0.950±0.029[a]	1.556±0.017	0.883±0.105	1.333±0.167
Gly (0.050)	93	6	1	0	108	100	0.900±0.053	0.833±0.046[a]	1.576±0.015	0.750±0.111	1.250±0.1112
Gly (0.025)	68	5	0	0	78	73	0.650±0.048	0.608±0.035[a]	1.585±0.010	0.667±0.105	1.167±0.105
Gly (0.0125)	39	0	0	0	39	39	0.325±0.021	0.325±0.021	1.589±0.008	0.583±0.083	1.167±0.167

BNCs, Binucleated cells; *MNi*, micronuclei; *MNC*, cells with 1 or more micronuclei; *CBPI*, Cytokinesis-Block Proliferation Index; *NPBs*, nucleoplasmic bridges; *NBUDs*, nuclear buds; *S.E.*, Standard Error; *NC*, Negative Control; *MMC*, Mitomycin-C; *Gly*, Gliphosate

[a] $P = 0.004$ (significantly differs from the DMSO solvent control, Mann-Whitney test)

Discussion

Glyphosate is an active ingredient of most widely used herbicides. Although it is believed to be less toxic than other herbicides, data about its possible genotoxicity are controversial and IARC classified this compound as probably carcinogenic to human (IARC 2015). The genotoxic effects of high concentrations of glyphosate have been documented, although with contradictory results, in a great number of scientific papers, as well as in evaluation reports of different international agencies. On the other hand, the effects of low concentrations of this compound, likely to be encountered in everyday life, were poorly investigated. Results of our study provided information about in vitro clastogenic effects of glyphosate on human lymphocytes at the low ADI concentration of 0.500 μg/mL and its submultiples. Based on the obtained data, it can be concluded that glyphosate significantly increased the CA and MNi levels in human lymphocytes at the ADI concentration of 0.500 μg/mL established by EFSA and at its submultiple concentrations, up to 0.025 μg/mL. The mechanisms underlying genotoxic potential of glyphosate alone or in complex with other compounds are unknown, although the exposure to glyphosate was found to trigger oxidative processes involved in the increase of the genomic damage. NPB frequency was found to increase with increasing glyphosate concentrations, although a statistical significance was found only at the higher glyphosate concentration. However, we obtained a significant linear regression due to a steady increase with the dose, indicating a possible effect of the compound inducing this kind of damage, which is consistent with the increased dicentric frequency observed in the chromosomal aberration test. Also, other authors analyzed in vitro the genotoxic potential of glyphosate in lymphocytes, but at exposure levels of higher magnitude orders. For example, in human lymphocytes cultured without S9 and in the presence of glyphosate at concentrations of 3.5, 92.8, and 580 μg/mL, the authors observed a slightly increased frequency of MNi and a significant tail length increase after a comet assay. Other authors evaluated the induction of CAs and MNi in blood cells of other animal models. Positive clastogenic and genotoxic effects of glyphosate on bovine peripheral lymphocytes cultured in vitro with herbicide concentrations ranging from 17 μM (2.874 μg/mL) to 170 μM (28.740 μg/mL) were reported, whereas another study reported no CAs effect of glyphosate at concentrations ranging from 28 (4.734 μg/mL) to 1120 μM (189 μg/mL). Contradictory results were obtained by a further study, in which observed, after 48 h of treatment without S9, a statistically significant increase in MNi frequency at 280 μM (47.34 μg/mL) but not at 560 μM (94.68 μg/mL) of glyphosate in one donor, and the opposite in a second donor (positive at 560 μM but not at 280 μM). Finally, another study in in vitro experiments based on comet assay, showed that 7 mM of glyphosate (1183 μg/mL) caused DNA damage in blood cells of Nile tilapia (Oreochromis niloticus). Concentrations of glyphosate similar to those evaluated in the present paper were tested in HepG2 cells by the MNi assay. Similarly to what we observed in human lymphocytes, these authors found a significantly higher number of MNi at the ADI value of 0.500 μg/mL, as well as at the residential exposure level of 2.91 μg/mL, after 4 h of treatment. Vice versa, negative results on Hep-2 cells were obtained with CA assay at glyphosate concentrations of 0.20 mM (33.8 μg/mL), 1.20 mM (203 μg/mL), and 6.00 mM (1014 μg/mL). Significant levels of DNA damage were also observed in human buccal epithelial cells exposed to glyphosate concentrations ranging between 10 and 20 mg/L, whereas another study showed that, in peripheral blood mononuclear cells, glyphosate induces DNA damage in the concentration range from 0.5 mM (84.54 μg/mL) to 10 mM (1690 μg/mL), and a significant decrease of global DNA methylation at concentration of 0.25 mM (42.27 μg/mL). Interestingly, the same authors also observed a significantly increased methylation of p53 promoter at concentrations of 0.25 mM and 0.5 mM (42.27 and 84.54 μg/mL). This hypermethylation was found to be able to downregulate the p53 gene expression and to activate proto-oncogenes, with consequent genomic alterations and cancer risk. The possibility of glyphosate causing cancer promotion in skin cells and proliferation in breast cells has been also observed in vivo and in vitro studies by mouse and human models, respectively. In this scenario, the results obtained in the present study require attention. Indeed, increased CA and MNi frequencies in peripheral blood lymphocytes have been positively associated with increased cancer risk and early events in carcinogenesis, respectively. Moreover, it should be emphasized that, beyond the cases of intoxication where glyphosate content in blood was found to range from 0.6 to 150 μg/mL, in subjects who were indirectly exposed to this substance, glyphosate was found in blood at concentrations of 0.074 ± 0.028 μg/mL, a value about seven times lower with respect to the established ADI value, but in the range of concentrations we tested (from 0.5 to 0.0125 μg/mL). At the same time, the genotoxicity of a compound should not be evaluated only after single administrations in in vitro or in vivo systems, but

also, and especially, after chronic administration of the same compound, even at lower quantities than those established by the competent agencies. In this sense, the clastogenicity we observed at concentrations of 0.100, 0.050, and 0.025 μg/mL represents an important signal, especially in view of a chronic exposure to these glyphosate concentration levels. Finally, no significant differences in CBPI and MI values were found between all tested concentrations and the solvent control, indicating that, at these concentrations, glyphosate does not influence in a significant manner the replicative capacity of the cells. These data differ from a study which observed a reduction of mitotic and proliferation indices in bovine lymphocytes, but at higher glyphosate concentrations (94.68 μg/mL and 189 μg/mL). Similarly, other authors described decreased levels of MI for other herbicides or insecticides, also in this case, at concentrations much higher than those tested in the present work.

Conclusion

In the present work, there was evidence for cytogenetic effects of glyphosate on cultured human lymphocytes. Despite the limitations of an in vitro study due to the reduced sample size, it is our opinion that the increased cytogenetic damage observed by our group at glyphosate concentrations equal and lower than the established ADI value requires further investigations in order to establish the effective genotoxicity threshold of this extensively used compound. Indeed, the glyphosate concentrations tested in the present work represent more realistic concentrations, likely to be encountered in everyday life, with respect to the higher doses evaluated in other published papers. In this scenario, in order to draw conclusions about the effects associated to the chronic exposure to low doses, in vitro studies are useful tools to investigate the dose response effects, the molecular mechanisms of action of different environmental xenobiotics, and their genotoxicity. This last, compared to other types of toxicity, may result in severe consequences that can be also inherited after long periods following exposure. The same DNA damage that occurs in a single cell, caused by low but chronic exposure to genotoxic compounds, can cause unexpected severe consequences in the long run.

Assessment and conclusion

Assessment and conclusion by applicant:

This paper describes human lymphocyte chromosome aberration and micronucleus tests with glyphosate. Although broadly compliant with OECD 473 and 487 there are some critical deficiencies which will have adversely influenced the reliability of the findings. Treatment with glyphosate was initiated 24 hours after lymphocyte cultures were stimulated to divide, instead of the recommended 48 hours, consequently the cultures would not have been asynchronous. This could mean cells in some stages of the cell cycle may have been under-represented, whilst others over-represented. Exposure to glyphosate was continuous for 28 hours in the chromosome aberration assay or 48 hours in the micronucleus assay. In contrast OECD test guidelines recommend maximum exposure of 1.5 cell cycles, equivalent to approximately 24 hours for lymphocyte cultures. For both endpoints the paper does not confirm if the slides were coded prior to analysis. The positive control has been compared statistically to the glyphosate treated cultures rather than the solvent controls.

The authors consider that glyphosate induces tri-tetraradial aberrations (amongst other aberration types) but fails to comment that the frequency of these aberrations observed at a single glyphosate concentration is 3-fold lower than the frequency observed in the solvent control cultures. Furthermore, it is unusual that the only multi-aberrant metaphases observed were a small number of positive control metaphases and gaps did not appear to increase with treatment but chromatid and chromosome breaks did.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Santovino *et al*., 2018	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		

Study in accordance to valid internationally accepted testing guidelines	Y	In accordance with OECD TG.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity is not reported. Source: Sigma-Aldrich, Saint Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	Lymphocytes.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	0.500, 0.100, 0.050, 0.025 and 0.0125 µg/mL.
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterized and although the genotoxicity tests conducted were in general in accordance with the OECD test guidelines, significant deficiencies were noted.		

Assessment and conclusion by RMS:
It is agreed with the applicant's assessment of the limitations of this publication. In contrast to the applicant, based on these limitations (as stated by the applicant above and by the RMS under 'deviations') the RMS considers the study to be supportive only.
The RMS considers both the CA and the MN assay as positive under the circumstances of the study. At concentrations of 0.025 to 0.5 µg/mL the study showed a dose-dependent increased frequency of micronuclei in the human peripheral lymphocytes cultured in the presence of different glyphosate concentrations. Also, glyphosate induced a dose-dependent increase structural chromosome aberrations including chromatid and chromosome breaks, dicentric chromosomes and acentric fragments. However, considering the methodological limitations, this outcome should be treated with caution.

B.6.4.4.7. *Genotoxicity – public literature, study 7*

Data point:	CA 5.4/007
Report author	Kasuba, V. *et al.*
Report year	2017
Report title	Effects of low doses of glyphosate on DNA damage, cell proliferation and oxidative stress in the HepG2 cell line
Document No	DOI 10.1007/s11356-017-9438-y E-ISSN: 1614-7499
Guidelines followed in study	Alkaline comet assay: Largely according to OECD GL 489, however, it is noted that the OECD GL describes *in vivo* Comet assays, whereas an *in vitro* Comet assay was performed within this

	publication; the Comet assay itself, however, was conducted similarly to the procedure described in OECD GL 489. For all other assays, no guideline was followed.
Deviations from current test guideline	- Alkaline comet assay: Duplicate slides were prepared per dose level, whereas (for in vivo comet assays) according to OECD GL 489 at least 5 analysable animals per dose group are required. In addition, a total of 100 nucleoids (50 per slide) were scored whereas OECD GL 489 requires at least 150 cells per animal to be scored. - HCD not reported; proficiency of lab not demonstrated - Purity not reported, test substance stability and test concentration not analytically verified.
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is supportive due to the deviations and limitations noted above and under "Assessment and conclusion RMS", respectively.

Full summary of the study according to OECD format

The toxic effects of glyphosate *in vitro* on HepG2 cells exposed for 4 and 24 h to low glyphosate concentrations likely to be encountered in occupational and residential exposures [the acceptable daily intake (ADI; 0.5 µg/mL), residential exposure level (REL; 2.91 µg/mL) and occupational exposure level (OEL; 3.5 µg/mL)] were studied. The assessments were performed using biomarkers of oxidative stress, CCK-8 colorimetric assay for cell proliferation, alkaline comet assay and cytokinesis-block micronucleus (CBMN) cytome assay. The results obtained indicated effects on cell proliferation, both at 4 and 24 h. The levels of primary DNA damage after 4-h exposure were lower in treated vs. control samples, but were not significantly changed after 24 h. Using the CBMN assay, a significantly higher number of MN and nuclear buds at ADI and REL after 4 h and a lower number of MN after 24 h were found. The obtained results revealed significant oxidative damage. Four-hour exposure resulted in significant decrease at ADI [lipid peroxidation and glutathione peroxidase (GSH-Px)] and OEL [lipid peroxidation and level of total antioxidant capacity (TAC)], and 24-h exposure in significant decrease at OEL (TAC and GSH-Px). No significant effects were observed for the level of reactive oxygen species (ROS) and glutathione (GSH) for both treatment, and for 24 h for lipid peroxidation. Taken together, the elevated levels of cytogenetic damage found by the CBMN assay and the mechanisms of primary DNA damage should be further clarified, considering that the comet assay results indicate possible cross-linking or DNA adduct formation.

Materials and Methods

Chemicals - Glyphosate was purchased as analytical standard purity grade (≤100%) as Pestanal®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Cell line - HepG2 cell line (ATCC® HB8065™) was purchased from the American Type Culture Collection (Rockville, MD, USA), cultivated in EMEM supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin-streptomycin (10,000 U/mL) and maintained in a humidified atmosphere (95 % relative humidity) with 5 % CO_2 at 37 °C. Testing was performed when the duplication time has been established to be constant, and the passage step was more than 3 but less than 10 cell passages.

Treatment conditions - The cells in culture were exposed to glyphosate concentrations of 0.5 µg/mL, 2.91 µg/mL and 3.5 µg/mL in PBS corresponding with the systemic concentrations at the acceptable daily intake (ADI), the residential exposure level (REL) and the occupational exposure level (OEL). The calculation of the concentrations to be tested was based on the average male human body weight of 60 kg and the total volume of 36 L of extracellular liquid. HepG2 cells were grown until 80 % confluence, trypsinized and transferred in cell culture flasks for the micronucleus and comet assays and in 96 well plates for the cell proliferation assay and the determination of markers of oxidative stress. Prior to treatment, the culture medium was removed, cells were washed with PBS and fresh complete medium with glyphosate was added. Cells were treated for 4 or 24 hours at 37 °C in a humidified atmosphere (95 % relative humidity) with 5 % CO_2. Negative and positive controls were tested in parallel.

Cell proliferation assay - Proliferation of HepG2 cells after 4 and 24 hours of exposure to glyphosate was studied by means of the CCK-8 colorimetric assay, based on the use of Cell Counting Kit-8. This kit uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, sodium salt (WST-8) that is bioreduced by cellular dehydrogenases to an orange formazan product, soluble in tissue culture medium in the presence of the electron carrier, 1-methoxy PMS. The amount of formazan produced is directly proportional to the number of living cells. At the end of treatment cells were washed with PBS and 10 µL of WST-8 solution added. After 4 and 24 hours of incubation at 37 °C in a CO_2 incubator, the optical density at 450 nm was determined for each well using a Victor3™ Multilabel Plate Reader. Data were expressed as a percentage absorbance compared to relevant negative controls. The positive control was 10 % DMSO in the complete nutrient medium. The experiment was done twice independently with 4 repeated measurements in each.

Alkaline comet assay - After 4 or 24 hours of treatment, cells were washed twice with PBS, detached by trypsinization, centrifuged and resuspended in complete EMEM. 7 µL of single cell suspension at 10^4 cells/mL was mixed with low melting point agarose and layered on slides. Duplicate slides were prepared for each time period and concentration together with negative and positive controls. Hydrogen peroxide was used as the positive control. Following preparation of all microgels, they were immersed in fresh cold lysis solution for 24 hours. Afterwards, slides were washed with bidistilled water and denatured in freshly prepared cold denaturation/electrophoresis buffer. After 20 min at 4 °C, cells were placed in the electrophoretic chamber and subjected to electrophoresis. Afterwards the slides were neutralized, dehydrated and stained with ethidium bromide (20 µg/mL) for image analysis with the fluorescent microscope Olympus BX51 (×200 magnification) using the Comet Assay IV software. A total of 100 nucleoids (50 per slide) were measured per each experimental point. The level of DNA damage was estimated based on tail intensity (TI), indicating %DNA in the comet tail. The experiment was performed once with two replicates that were compared, and if no statistically significant difference was found they were combined.

Cytochalasin B-blocked micronucleus cytome assay (CBMN cytome assay) - Cells were seeded at 10^4 cells/mL in complete EMEM medium. After 4 or 24 hours of treatment with glyphosate, cells were washed twice with PBS and fresh complete medium was added. At the 44^{th} hour, cytochalasin B (3 µg/mL) was added and the cell cultures harvested 24 hours thereafter. The positive control, cyclophosphamide, was incubated simultaneously. After harvesting, the medium was discarded, and cells were washed twice with PBS, detached by trypsinization, rinsed and resuspended in complete EMEM medium. Cell suspensions in complete EMEM were centrifuged and the pellet resuspended in PBS, centrifuged and the pellet fixed and put on slides for staining with 2 % Giemsa stain and air-dried. The experiment was performed once with two replicates. Micronuclei (MNi), nucleoplasmic bridges (NPBs), nuclear buds (NBUDs) and apoptotic and necrotic cells were scored in binucleated (BN) cells. A total of 2000 BN cells per each experimental point were scored to determine the parameters of the CBMN Cytome assay.

Lipid peroxidation - Malondialdehyde (MDA), and endproduct of lipid peroxidation, was measured using the thiobarbituric acid reactive substance (TBARS) assay with some modification. 0.5 mL of sample was added to 0.5 mL of thiobarbituric-trichloroacetic acid (TBA-TCA) reagent and heated at 90 °C for 30 min and then cooled in an ice bath and centrifuged. Absorbances were measured with a microplate spectrophotometric system (Victor3™ Multilabel Plate Reader) at 530 nm. Two independent experiments were performed and each sample was measured in duplicate. The TBARS concentration in unknown samples was calculated using a standard curve constructed with 1,1,3,3- tetrametoxypropane (0.3–6.07 μM) and expressed as μM.

Total antioxidant capacity - Total antioxidant capacity (TAC) was investigated using the ferric-reducing ability of plasma (FRAP) assay, based on the reduction of Fe^{3+}-TPTZ complex under acidic conditions. The FRAP assay used in this study was slightly modified. 100 μL of cell suspension (10^4 cells in total) was added to 1.0 mL of FRAP reagent. Absorbance was measured after 4 minutes of incubation at 593 nm using the FRAP working solution as a blank. Two independent experiments were performed and each sample was measured in duplicate. The results were calculated on a basis of a standard curve using $Fe_2SO_4 \cdot 7H_2O$ (0.05-4.0 mM).

ROS detection - The amount of intercellular reactive oxygen species (ROS) was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). DCFH-DA is deacetylated by cellular esterases to form a non-fluorescent compound which is then oxidized in the presence of hydroxyl, peroxyl and other ROS to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). All measurements were performed in quadruplicate in dark-sided 96-well microplates in which each well was added 100 μL of cell suspension containing 10^4 cells. Cells were grown in the same medium and conditions as for the cell proliferation assay and treated with the same concentrations of glyphosate for 4 and 24 hours. After treatment the cells were washed with PBS and 100 μL of 50 μM DCFH-DA dye diluted in PBS was added and kept in contact with the cells for 30 minutes at 37 °C in a CO_2 incubator. The control for dye autofluorescence was prepared without addition of dye. Negative (non-treated) cell controls in EMEM were included in each experiment. The positive control was a solution of 100 mM H_2O_2. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Data were expressed as fluorescence arbitrary units (AUs) and later transformed into percentages compared to control values.

Quantification of glutathione - The fluorogenic bimane probe, monochlorobimane (MBCl), reacts specifically with glutathione (GSH) and forms a fluorescent product equal to the amount of GSH that can be measured fluorometrically. Cell cultures in 96-well plates were prepared with 100 μL of cell suspension containing 10^4 cells and each well was treated with glyphosate for 4 and 24 hours. After washing with PBS, cells were incubated with 100 μL of 50 μM MBCl in PBS for 20 minutes at 37 °C in a CO_2 incubator. Measurements were made using a Victor3™ Multilabel Plate Reader at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Negative cell controls were included in each experiment. Data were expressed as fluorescence AUs and later transformed into percentages compared to control values. All of the measurements were performed in quadruplicate.

Glutathione peroxidase activity - Glutathione peroxidase (GSH-Px) activity in HepG2 cells was measured according to the European standardized method. To increase assay sensitivity for the measurement of GSH-Px, samples were prediluted 60 times instead of a recommended 110-fold dilution as used for blood samples. The amount of glutathione oxidized by *t*-butyl hydroperoxide was determined by following the decrease in β-NADPH concentration at 340 nm. Two independent experiments were performed and each sample was measured in duplicate. One unit of GSH-Px was expressed as the amount of enzyme that oxidizes 1 μmol β-NADPH/min at 37 °C. Activity of GSH-Px was expressed in U/g total protein. The protein concentration of cell lysates was measured using a Total Protein Kit, Micro. Samples, standards and blanks were tested in triplicate, and absorbance was measured in 96-well plates at 570 nm**.**

Statistical analysis - Statistical analysis was performed using StatSoft Dell Statistica 13 package program. Descriptive statistics were used to determine the basic statistical parameters (mean, standard error and deviation, median and minimum and maximum values). Data gathered with the Comet Assay IV software were logarithmically transformed prior to statistical evaluation, with the aim of normalizing distribution and equalizing variances. The intra- and intergroup comparisons between samples were performed using one-way analysis of variance (ANOVA) with post hoc Scheffé's test. Comparisons between values obtained with the CBMN Cytome assay and HepG2 proliferation kinetics measured with CBMN were made by Pearson's $\chi 2$ test for two-by-two contingency tables. For the biochemical and cell proliferation assays descriptive statistics and *t* test for comparisons between independent samples were used. The level of statistical significance was set at $p < 0.05$.

Results

Cell proliferation measured by the CCK-8 assay - Although without statistical significance a slight increase of cell proliferation was observed in HepG2 cells after a 4-hour treatment of about 9 % at the ADI and almost 8 % at the REL. The values recorded for the OEL-treated cells were not different from control. After 24 hours of treatment, HepG2 cells showed a not statistically significant increase in cell proliferation of 3 % at the REL and of 1 % at the OEL when compared to the control. Proliferation of ADI-treated cells did not differ from control.

Primary DNA damage measured by the alkaline comet assay - After 4 hours of exposure, the TI was statistically significantly decreased at all glyphosate concentrations compared to the negative control, whereas the TI of the positive control was significantly higher than the negative control. After 24 hours of exposure, the TI at ADI, OEL and REL were not statistically significantly different from control. Throughout comet measurements, apoptotic and necrotic nucleoids were also counted but there were no significant deviations from control.

Cytogenetic damage measured using CBMN cytome assay - After 4 hours of exposure, a not statistically significant increase in MN was found in HepG2 cells treated with glyphosate at all 3 concentrations. In HepG2 cells treated with glyphosate at ADI and REL, a statistically significant increase in nuclear buds (NBUD) frequency was observed. The incidence of nucleoplasmic bridges (NPB) was highest in cells treated with glyphosate at ADI (6 per 2000 BN cells). Only 2 apoptotic cells per 2000 cells were found in the sample treated with glyphosate at OEL. The nuclear division index (NDI) in glyphosate-treated cells was different from that of the control. In the OEL-treated sample, there were statistically significant changes in the distribution of M1–M4 cells and OEL treatment resulted with more cells in the M2 phase. The same effect was observed when ADI-treated and OEL-treated samples were compared, as well as when REL-treated and OEL-treated samples were compared. After 24 hours of exposure, a lower number of micronucleated BN cells and a lower frequency of BN MN was found at all 3 glyphosate concentrations when compared to the control. Also the frequency of BN NBUDS was statistically significantly lower at all 3 concentrations. Control and glyphosate-treated cells did not significantly differ in the number of NPBs. At ADI and REL, seven and five apoptotic cells per 2000 BN cells were found, respectively. There was no statistically significant difference in NDI values between glyphosate-treated samples and control. A slightly changed incidence of M2 and M3 cells (i.e. cells in the S and G2+M phase of cell cycle, respectively) was found in treated samples as compared to control.

Lipid peroxidation - After 4 hours of exposure, TBARS concentrations in samples treated with glyphosate at ADI and OEL were statistically significantly lower than control values. Treatment of HepG2 cells for 24 hours with glyphosate resulted in lower TBARS concentrations at REL, but the difference was not statistically significant.

GSH level - The level of GSH was not statistically significantly different from the control value for all concentrations and exposure times. After 4 hours of treatment, the ADI-treated sample showed a 4 % higher level of GSH than the control whereas REL and OEL-treated cells showed GSH levels similar to control values. The 24-hour ADI-treated sample had the lowest GSH concentration, which was 5 % lower than the levels measured in the control samples, and REL- and OEL-treated cells showed GSH levels similar to control values.

GSH-Px activity - GSH-Px activity was statistically significantly decreased in HepG2 cells exposed to glyphosate at the ADI concentration after 4 hours. After 24 hours of treatment, a statistically significant decrease in the GSH-Px activity was observed at the OEL concentration.

Discussion

The present study evaluated how low-dose exposure of glyphosate for 4 and 24 hours affected cytotoxic, genotoxic and biochemical parameters in the human hepatoma cell line HepG2. Glyphosate increased not statistically significantly cell proliferation in HepG2 cells after 4 and 24 hours of exposure. The effect was more pronounced after 4 hours (8–9 % as compared to control), than after 24 hours (3 % as compared to control). The results of this study have demonstrated that exposure to glyphosate for 4 hours led to a slightly increased cytogenetic damage in terms of MN. Despite the non-significant increase in MN frequencies at ADI and REL, significant increases in nuclear bud frequency were found after 4 hours of exposure. Since nuclear budding represents a mechanism of MN formation, these results indicate that even a low dose of glyphosate as the ADI influences the level of DNA damage and cell stability. After 24 hours of exposure, all of the treated cells showed a lower frequency of micronuclei, nuclear buds and nucleoplasmic bridges than the control. The results obtained suggest that glyphosate exerts the highest DNA-damaging potential after 48 or even after 72 hours of treatment. Considering that the shorter exposure lasted only 4 hours, while the total cell culture lasted for 72 hours before harvesting, there was an opportunity to better examine the effects of low-dose exposure on DNA damage. On the contrary, in the case of an exposure of 24 hours it seems that this effect only started to be slightly visible. Serum in the cell culture can mask the development of DNA damage observed in micronucleus assay results. This can be the explanation, why the standard time period used in this experiment did not demonstrate an effect, while in the comet assay the primary DNA damage demonstrated higher damage levels.

Conclusion

All cytogenetic, molecular and biochemical methods used in this study indicate that glyphosate applied at low concentrations, possesses a toxic potential towards HepG2 cells, which has to be further explained. It seems that the theory of different effects of low-dose vs. high-dose exposure, and more deleterious effects at low doses, is true. Although at ADI, REL and OEL, no drastically different levels of oxidative damage were seen, the elevated level of permanent DNA damage found with the micronucleus assay calls for concern, especially if leading to adduct formation, as shown by comet assay results. Based on the obtained results, it cannot be said without doubt whether glyphosate acts as an aneugen or a clastogen, but there are indications from previous studies that the aneugenic effect plays an important role in the formation of micronuclei. This study did not evaluate the effects of glyphosate on cytoskeleton and proteins, but this would be a direction for future evaluations of glyphosate toxicity, together with the clarification of its effects on cell membrane level, especially in different phases of the cell cycle.

Assessment and conclusion

Assessment and conclusion by applicant:

The objective of this study was to examine the effects of acute exposure (4 and 24 hours) of human hepatoma HepG2 cells to glyphosate at concentrations equivalent to the systemic concentrations at the ADI, REL and OEL. The endpoints investigated in this study are cell proliferation, DNA damage, MN formation and oxidative stress. A non-statistically significant increase in cell proliferation was seen in the CCK-8 test with no dose-effect relationship. The comet assay showed a statistically significant decrease in tail intensity after 4 hours with no difference from control after 24 hours. In the CBMN cytome assay a non-statistically significant increase in BN MN frequency was seen after 4 hours without a dose-effect relationship. After 24 hours, a decrease instead of an increase in BN MN frequency was reported. The nuclear bud frequency was statistically significantly elevated after 4 hours of exposure but was statistically significantly lower than control after 24 hours of exposure. The indicator tests for oxidative stress did not show a substance related effect.

Overall, the results of the study do not indicate a genotoxic potential of glyphosate. The lack of statistical significance, reproducible effects as well as the fact that the control values in the Comet assay and micronucleus assay seem to be highly variable limit the reliability of the study.

This publication is considered relevant but reliable with restrictions because the cytogenetic damage found *in vitro* at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Kasuba *et al.*, 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing		

guidelines		
Study performed according to GLP		
Study completely described and conducted following scientifically acceptable standards		
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≤100 % as Pestanal®. Source: Sigma-Aldrich Laborchemikalien GmbH, Germany.
Only glyphosate acid or one of its salts is the tested substance		As "Pestanal"
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	Culture concentration not in correspondence with ADI of 0.5 mg/kg bw.
Metabolic activation system clearly and completely described	N	HepG2 cells used
Test concentrations in physiologically acceptable range (< 1 mM)	Y	
Cytotoxicity tests reported	N	Very low concentrations used
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	?	No significant effects were observed for the level of ROS and GSH for 4h and 24h incubation, and for 24 h for lipid peroxidation. Elevated levels of cytogenetic damage were found in the CBMN assay and the comet assay results indicate possible cross-linking or DNA adduct formation. These data were obtained at in vitro test concentrations that correspond with an external dose of 0.5 mg/kg bw/day (ADI) whereas there are regulatory studies with no effects at doses up to 2,000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because the cytogenetic damage found in vitro at a systemic concentration corresponding with the ADI (0.5 µg/mL which should have been 0.17 µg/mL) was not confirmed in *in vivo* regulatory MN studies with doses up to 2000 mg/kg bw corresponding with a systemic concentration of about 50 µg/mL.		

Assessment and conclusion by RMS:

It is agreed with the applicant's summary of the study as described above.
The RMS adds that a decrease in tail intensity might indicate DNA cross-links, however, according to OECD TG 489 this cannot be reliably detected with standard experimental conditions.

In contrast to the applicant, however, the study is considered supportive only by the RMS due to the reported limitations of the study. Amongst others, test were not performed with and without metabolic activation (HepG2 cell lines are known to have a low expression and activity of CYP enzymes). Besides, many of the experiments were performed in duplicate only. It may therefore be questionable whether this provides an adequate statistical power. It is also agreed with the applicant that the reliability if further limited by "the lack of statistical significance, reproducible effects as well as the fact that the control values in the Comet assay and micronucleus assay seem to be highly variable."

For the sake of completeness, results of the comet assay and the CBMN cytome assay in tabulated form were included by the RMS below since the applicant did not include any tables or figures in its summary.

Table B.6.4.4.7-1: Results of the alkaline comet assay TI parameter on HepG2 cells treated for 4 and 24 h with glyphosate applied at three concentrations

C	ADI	REL	OEL	PC
4 h (TI)				
1.45 ± 2.43 (0.24)	0.16 ± 0.27 (0.03)[a]	0.20 ± 0.33 (0.03)[a]	0.16 ± 0.26 (0.03)[a]	16.96 ± 13.49 (1.35)[a]
0.56	0.03	0.06	0.04	13.72
0–18.47	0.00–1.12	0.00–1.61	0.00–1.36	0.18–47.59
24 h (TI)				
0.06 ± 0.10 (0.01)	0.06 ± 0.10 (0.01)	0.11 ± 0.16 (0.02)	0.05 ± 0.09 (0.01)	54.49 ± 22.05 (2.20)
0.02	0.00	0.02	0.01	61.46
0.00–0.51	0.00–0.46	0.00–0.62	0.00–0.43	2.32–84.68

Negative and positive controls were studied in parallel. Data are reported as mean ± SD (SE) (first row), median (second row) and range (third row); $p < 0.05$

C control (EMEM), *PC* positive control (50 µM H_2O_2), *ADI* acceptable daily intake, *REL* residential exposure level, *OEL* occupational exposure limit, *TI* tail intensity (parameter of comet assay; %DNA in comet tail)

[a] Significantly different than control

Table B.6.4.4.7-2: Results of CBMN cytome assay on HepG2 cells treated for 4 and 24 h with glyphosate applied at three concentrations

Parameter	Total MN	BN MN	Distribution of BN cells according to No. of MN				BN MN‰ ± SD	BN NPBs‰ ± SD	BN NBUDs‰ ± SD
Treatment			0	1	2	3			
4 h									
C	13	13	1987	13	–	–	6.5 ± 2.12	2.0 ± 1.41	20.0 ± 1.41
ADI	20[a]	20	1980	20	–	–	10.0 ± 2.83	3.0 ± 0	36.5 ± 2.12[a]
REL	21[a]	20	1980	19	1	–	10.0 ± 2.83	2.5 ± 0.71	37.5 ± 7.78[a]
OEL	28[a]	25	1975	22	3	–	12.5 ± 2.12	2.5 ± 0.71	27.0 ± 11.31[b]
PC	58[a]	49	1951	42	5	2	24.5 ± 3.54[a]	3.5 ± 0.71	16.5 ± 3.54
24 h									
C	34	34	1966	34	–	–	17.0 ± 1.41	0	39.0 ± 0
ADI	28	26	1974	24	2	–	13.0 ± 4.24[b]	1.0 ± 0	21.5 ± 6.36[a]
REL	19[a]	16	1984	13	3	–	8.0 ± 2.83	1.0 ± 0	4.5 ± 2.1[ab]
OEL	9[ab]	9	1991	9	–	–	4.5 ± 2.12	1.0 ± 0	6.0 ± 1.41[ab]
PC	51[a]	46	1954	41	5	–	23.0 ± 2.83[a]	9.5 ± 2.12	28.0 ± 4.24[a]

Negative and positive controls were studied in parallel. All data are presented as mean ± SD of two analyses (one experiment, two replicates that were analysed separately, and if no difference was found, were merged and analysed together). A total of 2000 cells were scored per concentration for the MN and other nuclear anomalies in binuclear cells, $p < 0.05$

C negative control (EMEM), *PC* positive control (0.28 mg/mL cyclophosphamide), *MN* micronucleus, *BNMN* total number of binucleated cells with MN, *BNNPB* binucleated cells with nucleoplasmic bridge, *BNNBUD* binucleated cells with nuclear bud, *ADI* acceptable daily intake, *REL* residential exposure level, *OEL* occupational exposure limit

[a] Significantly different from negative control

[b] Significantly different from ADI

B.6.4.4.8. *Genotoxicity – public literature, study 8*

Data point:	CA 5.4/008
Report author	Kwiatkowska, M. *et al.*
Report year	2017
Report title	DNA damage and methylation induced by glyphosate in human peripheral blood mononuclear cells (in vitro study)
Document No	doi.org/10.1016/j fct.2017.03.051 ISSN: 0278-6915
Guidelines followed in study	None (OECD GL 489 only describes *in vivo* comet assays, whereas an *in vitro* comet assay was performed within this publication; the comet assay itself, however, was conducted similarly to the procedure described in OECD GL 489)
Deviations from current test guideline	– Number of donors analysed in the genotoxicity test (3) not in line with OECD GL 489 (at least 5 animals). It is noted, however, that the number of scored cells per donor is in line with the OECD GL (150 cells) – No information provided regarding metabolic activation. Considering that it is an *in vitro* assay, test conditions with and without metabolic activation should be investigated. – No HCD are available; lab proficiency not proven. – Cell viability was determined after isolation of PBMCs and after exposure to glyphosate, however, the exact method to assess cell viability is not given.
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive.

Full summary of the study according to OECD format
The purpose of this study was to assess DNA damage (determination of single and double strand-breaks by the comet assay) as well as to evaluate DNA methylation (global DNA methylation and methylation of p16 (CDKN2A) and p53 (TP53) promoter regions) in human peripheral blood mononuclear cells (PBMCs) exposed to glyphosate. PBMCs were incubated with the compound studied at concentrations ranging from 0.1 to 10 mM for 24 h. The study has shown that glyphosate induced DNA lesions, which were effectively repaired. However, PBMCs were unable to repair completely DNA damage induced by glyphosate. A decrease in global DNA methylation level at 0.25 mM of glyphosate was also observed. Glyphosate at 0.25 mM and 0.5 mM increased p53 promoter methylation, while it did not induce statistically significant changes in methylation of p16 promoter. To sum up, it was shown for the first time that glyphosate (at high concentrations from 0.5 to 10 mM) may induce DNA damage in leucocytes such as PBMCs and cause DNA methylation in human cells.

Materials and methods
Chemicals – Glyphosate (95 % purity) commercially obtained from Sigma-Aldrich, USA.

Isolation of PBMCs - PBMCs were isolated from leucocyte-buffy coat from blood collected from 9 healthy volunteers (aged 18-55 years) with no symptoms of infectious disease. For each parameter, 3 leucocyte-buffy coats were taken from 3 blood donors. After dilution with PBS (1:4) PBMCs were isolated from the buffy coat by centrifugation using lymphocyte separation medium (LSM). The PBMCs were collected, suspended in erythrocyte lysis buffer and incubated. Afterwards PBS was added and the cells centrifuged. The supernatant was decanted and the cells were washed twice with RPMI with L-glutamine and 10 % fetal bovine serum (FBS). The cells were resuspended in RPMI medium with L-glutamine, 10 % FBS and penicillin-streptomycin (0.5 %) and counted in the hemocytometer. The final PBMCs density used in the experiments after addition of glyphosate was $1x10^6$ cells/mL. After incubation, PBMCs were diluted to a density of 5×10^4 cells/mL for the comet assay and condensed to a density of 5×10^6 cells/mL for the conduct of epigenetic methods. The viability of the cells was over 94 %.

Treatment of PBMCs - Glyphosate was dissolved in PBS and the final concentrations of glyphosate in the comet

assay were in the range of 0.25 to 10 mM, while epigenetic changes were assessed after exposure to glyphosate at 0.25 and 0.5 mM. For the determination of DNA damage the cells were incubated with glyphosate for 24 hours and repair of the DNA lesions was assessed after a recovery period of 120 minutes following incubation. In the comet assay, 0.25 mM was chosen as the lowest concentration of glyphosate which produced any statistically significant changes in DNA damage. Epigenetic changes were assessed at 0.25 mM which did not induce DNA damage and at 0.5 mM which induced statistically significant DNA damage. For the assessment of cell viability PBMCs were incubated for 24 hours with different concentrations of glyphosate. Afterwards, the cells were centrifuged and the test solution discarded. The cells were then resuspended in RPMI medium and incubated for an additional 2 hours. Cell viability was 90.0 %, 90.2 %, 90.4 %, 88.5 %, and 87.8 % for control, 0.5, 5, 7.5 and 10 mM of glyphosate, respectively. Each DNA damage experiment included hydrogen peroxide as the positive control.

Alkaline comet assay - The comet assay was performed under alkaline conditions with some modifications. A freshly prepared cell suspension in 0.75 % low melting point agarose dissolved in PBS was layered onto microscope slides pre-coated with 0.5 % normal melting point agarose. The cells were lysed for 1 hour at 4 ˚C in an alkaline buffer and DNA was allowed to unwind for 20 minutes in an alkaline solution. Electrophoretic separation was performed in an alkaline solution at 4 ºC for 20 minutes at an electric field strength of 0.73 V/cm (28 mA). Then, the slides were washed in water and stained with 2 mg/mL 4',6-diamidino-2-phenylindole (DAPI) and covered with cover slips. The comets were observed at 200 x magnification in an Eclipse fluorescence microscope. Fifty images (comets) were randomly selected from each sample and the mean value of DNA in the comet tail was taken as an index of DNA damage (expressed in percent). For one blood donor, 3 parallel tests with aliquots of the sample of the cells were performed for a total number of 150 comets. A total number of 450 comets from 3 blood donors was recorded to calculate mean ± SEM.

DNA repair - The control samples and the PBMCs treated with glyphosate at 0.5, 5, 7.5 and 10 mM were washed and resuspended in fresh RPMI 1640 medium with L-glutamine preheated to 37 ˚C. Aliquots of the suspension were taken immediately after incubation at time "zero" and 120 minutes later. The samples were placed in an ice bath to stop DNA repair. The repair was assessed as a decrease in the extent of DNA damage measured after 120 minutes of post-incubation using the alkaline version of the comet assay.

Methylation of p16 (CDKN2A) and p53 (TP53) promoter regions - Chemical modification of 500 ng of genomic DNA was performed with the Cells-to-CpG™ Bisulfite Conversion Kit. For methylation analysis, a quantitative methylation-specific real-time PCR assay (qMSP) was conducted in 3 independent experiments including 3 blood donors with FastStart SYBR Green Master. All samples were amplified in triplicate. To determine the methylation status of a particular gene expressed as the methylation index (MI) in percentage, the Ct values of the methylated gene of interest were compared with the Ct values of the unmethylated gene of interest.

Global DNA methylation - Global DNA methylation was determined by means of DNA quantification using 5-methyl cytosine (5-mC) monoclonal antibodies in an ELISA-like reaction using the Methylflash Methylated DNA Quantification Kit. DNA (100 ng) isolated from whole blood PBMCs was used for analysis. Each sample was analyzed in duplicate and the determination was repeated whenever there was a failure in detection. The calculation of the amount of 5-mC was done with the use of a standard curve. Methylation levels were calculated relative to the methylated control DNA and expressed as a percentage of methylated DNA.

Statistical analysis - The mean value was calculated for three independent experiments (3 blood donors), whereas for each individual an experimental point was a mean value of at least 2 (methylation analysis) or 3 replications. Statistical analysis was conducted using the Mann-Whitney test, the Student's t-test and one-way analysis of variance (ANOVA) with a post hoc multiple comparisons procedure. The differences were considered to be statistically significant when $p<0.05$.Data analysis was performed using STATISTICA software.

Results

Analysis of DNA strand-breaks and DNA repair - DNA damage (single and double strand-breaks and alkali-labile sites formation) was statistically significantly increased from a glyphosate concentration of 0.5 mM on. At 10 mM the statistically significant increase in DNA damage exceeded 13 times the control value. After 120 minutes of recovery significant repair was observed of the DNA lesions induced by glyphosate (5.61 % vs 2.93 %, 10.51 % vs 5.07 %, , 14.91 % vs 7.76 %, and 27.49 % vs 11.05 % at 0.5 mM, 5 mM, 7.5 mM, and 10 mM of glyphosate, respectively.

DNA methylation status - As compared to control cells, the percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM, but not at 0.5 mM. On the contrary, p53 promoter methylation was statistically significantly increased at both concentrations of glyphosate. Methylation of the p16 gene promotor was increased after treatment with glyphosate but this change was not statistically significant.

Discussion and Conclusions

This study showed that glyphosate induced DNA damage in PBMCs in the concentrations range from 0.5 to 10 mM. Moreover, we noticed that PBMCs significantly repaired glyphosate-induced DNA damage, but they were unable to repair completely DNA strand-breaks after 120 min post-incubation (2.23% (control) vs 2.93% (0.5 mM), 5.07% (5 mM), 7.76% (7.5 mM) and 11.05% (10 mM), respectively. Unrepaired DNA damage can lead to mutations that may cause genetic instability, and tumor growth.

This study revealed that glyphosate decreased global DNA methylation in PBMCs statistically significantly at a concentration of 0.25 mM. Surprisingly, the effect of glyphosate at 0.5 mM on global DNA methylation was not statistically significant although the mean value from three independent experiments was still decreased as compared to control. Along with the decreased global DNA methylation, a statistically significant increase in methylation of the p53 promoter region was observed at 0.25 and 0.5 mM. Altered p53 promoter hypermethylation is an epigenetic pattern frequently observed in human cancers. Thus, the results of this study suggest that glyphosate at high concentrations (≥ 0.25 mM) may cause a down-regulation of p53 gene expression and activate proto-oncogenes or retrotransposable sequences which may induce genomic alterations by insertion and/or homologous recombination. This study showed for the first time that glyphosate may induce DNA damage in human leucocytes and cause epigenetic alterations in animal cells.

Assessment and conclusion

Assessment and conclusion by applicant:

It was the objective of this study to investigate the effect of high glyphosate concentrations on DNA integrity and DNA methylation in PBMCs *in vitro*. It was demonstrated that glyphosate increased statistically significantly DNA damage (single and double strand-breaks and alkali-labile sites formation) from 0.5 mM up to 10 mM. Repair of the DNA lesions was significant at all concentrations tested after 120 minutes of recovery. The percentage of the global DNA methylation level was statistically significantly decreased by glyphosate at 0.25 mM but not at 0.5 mM. On the contrary, p53 promoter region methylation was statistically significantly increased as compared to control cells at 0.25 and 0.5 mM.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Kwiatkowska *et al.*, 2017.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95 %. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	Y	Salt not mentioned
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described		

Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.25 to 10 mM
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	For DNA damage. The concentration at which DNA damage was observed is higher than the blood concentrations in rats obtained after dosing at the limit dose of 2000 mg/kg bw for the detection of MN in vivo. The results obtained are not corroborated by regulatory in vivo genotoxicity studies.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the lowest concentration at which DNA damage was observed (0.5 mM) is higher than the blood concentrations in rats (0.3 mM) obtained after dosing at the limit dose of 2000 mg/kg bw where no MN effects were seen.		

Assessment and conclusion by RMS:

The effect of glyphosate on DNA integrity and DNA methylation was investigated in PBMCs *in vitro*. Glyphosate increased statistically significantly DNA damage at and above 0.5 mM, but it was also shown that DNA repair after 120 min of recovery was significant. In addition, global DNA methylation was statistically significantly decreased by glyphosate at 0.25 mM but not at 0.5 mM, whereas methylation of the p53 promoter region was statistically significantly increased compared to control cells at 0.25 and 0.5 mM. Methylation of the p16 promotor region was not statistically significant altered.

The study indicates statistically significant DNA damage but this effect seems only to occur at concentrations above that found *in vivo* in rats given 2000 mg/kg bw (i.e., 0.3 mM) and can, thus, be considered an irrelevant effect.

In contrast to the applicant, the study is considered supportive. Several deviations were noted from OECD guideline 489 with regard to the conduct of the alkaline comet assay (see above), although the RMS recognises that the OECD guideline 489 in fact only addresses *in vivo* studies. In addition, the description of the donors is rather vague ("healthy volunteers (aged 18-55 years) with no symptoms of infectious disease"), i.e. no indications about smoking, potential medication, alcohol consumption etc. is given. Therefore, the observations may also be confounded.

For the sake of completeness, results of the comet assay were included by the RMS below since the applicant did not include any tables or figures in its summary.

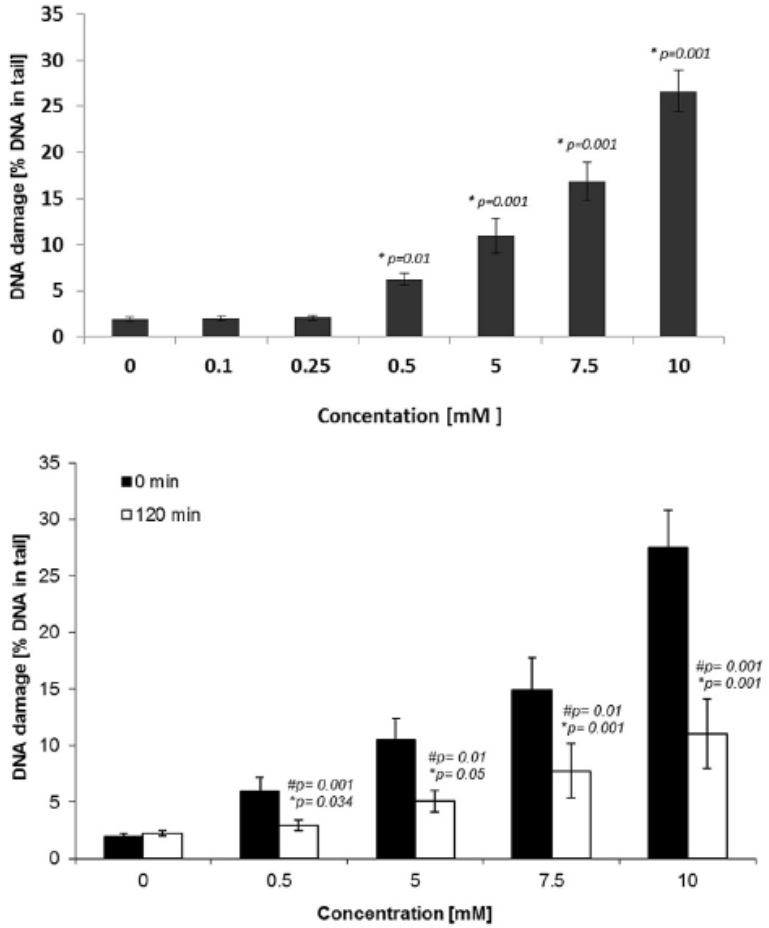


Figure B.6.4.4.8-1: A. DNA damage in human PBMCs incubated with glyphosate in the concentrations ranging from 0.1 to 10 mM for 24 h at 37 °C. Error bars denote SEM; *p value as compared with control. **B.** Time course of the repair of damaged DNA in human PBMCs after 24 h incubation with glyphosate in the concentrations ranging from 0.5 to 10 mM. The repair was assessed as a decrease in the extent of DNA damage measured after 120 min of post-incubation (the percentage of the DNA in comet tail) using the alkaline version of the comet assay. The results are mean of three independent experiments (donors). Error bars denote SEM, #p value as compared with the extent of DNA damage in time of 0 min,*p value as compared with control after 120 min.

B.6.4.4.9. *Genotoxicity – public literature, study 9*

Data point:	CA 5.4/009
Report author	Suárez-Larios, K. *et al.*
Report year	2017
Report title	Screening of Pesticides with the Potential of Inducing DSB and Successive Recombinational Repair
Document No	DOI: 10.1155/2017/3574840
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:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive

Full summary of the study according to OECD format
A study was realized to ascertain whether glyphosate and other selected pesticides would induce double strand breaks (DSB) in lymphocyte cultures and whether this damage would induce greater levels of proteins Rad51 participating in homologous recombination or of p-Ku80 participating in nonhomologous end joining. Only five pesticides were found to induce DSB of which only glyphosate and another one pesticide induced a significant increase of p-Ku80 protein, indicating that nonhomologous end joining recombinational DNA repair system would be activated. The type of gamma-H2AX foci (an indicator for double strands breaks) observed was comparable to that induced by etoposide (a medication used for the treatments of a number of types of cancer) at similar concentrations. These results are of importance since these effects occurred at low concentrations in the micromolar range, in acute treatments to the cells. Effects over longer exposures in actual environmental settings are expected to produce cumulative damage if repeated events of recombination take place over time.

The RMS notes that the summary presented below is focussed on glyphosate and AMPA, and not on the other substances since these are not considered relevant in the frame of the current application for renewal.

Materials and Methods
Chemicals - Endosulfan, glyphosate, pentachlorophenol, permethrin, propoxur, paraoxon, AMPA (glyphosate metabolite), endosulfan lactone (endosulfan metabolite), and etoposide (positive control) were obtained from Sigma-Aldrich, Mexico. Purity was not reported.

Evaluation of DNA double strand breaks (DSB)- Three mL of blood were obtained from fully informed healthy young male donors of 21 to 35 years old. The volunteers were nonsmokers, did not consume alcohol and had not taken medication or were not subjected to radiation for medical purposes. The concentrations of glyphosate tested were 0, 0.4, 2, 10, and 50 µM, and those of AMPA were 0, 40, 200, 1,000 and 5,000 µM. Whole blood was diluted in RPMI-1640 medium and treated with the test compound 1.5 hours at 37 ºC, after which 0.075 M KCl was added and the incubation continued for 30 minutes. Lymphocytes were recovered by centrifugation and formaldehyde added to reach a final concentration of 4 %. Then PBS and Triton X-100 were added followed by an incubation of 30 minutes. Thereafter the cells were washed, supplemented with 4 % fetal bovine serum and isolated by centrifugation and 1 mL of cold 50 % methanol added. After one night at −20 ºC the samples were centrifuged at 0 ºC, and cold methanol added to the cells which were kept at −20ºC until analysis. Treatments were done in duplicate. DNA double strand breaks were detected by means of immunofluorescence of phosphorylated histone H2AX foci. Staining of lymphocyte nuclei was done as follows: the slides were washed and blocked with KCMT buffer for 1 hour at room temperature. Primary antiphospho-histone H2AX (Ser139) in blocking solution was added and left to incubate overnight followed by washes with KCMT buffer. Then the cells were incubated with the secondary antibody Alexa-Fluor 555 goat anti-mouse for 1 hour at room temperature. The slides rinsed in deionized water before mounting in DAPI mounting medium and subsequently analyzed for γ-H2AX foci under a fluorescence microscope. Evaluation of foci was done in 2 slides per concentration and 50 cells were evaluated in 3 different regions per slide. In total, 300 cells were evaluated per treatment. When a nucleus presented one or more foci, it was considered positive. The extent of DNA damage was classified in 3 categories: percentage of cells without γ-H2AX foci (no DNA damage), with less than 10 γ-H2AX foci (moderate DNA damage) and with more than 10 γ-H2AX foci (severe DNA damage). Additionally, the damage was expressed as mean percentage of γ-H2AX positive nuclei.

Cytotoxicity - Cytotoxicity was tested using The CellTiter 96 AQueous One Solution Reagent from PROMEGA and following the manufacturer's instructions. Mononuclear cells were isolated from blood using Histopaque-1077. The cells were plated in well plates at 100,000 cells per well, and treated with the test compounds in triplicate for each concentration. Absorbance at 490 nm was recorded using a 96-well plate reader. The percentage of survival was calculated as (Absorbance at 490 nm of treatment/Absorbance at 490 nm of the negative control) × 100.

Western blot analysis of proteins participating in DNA recombination - Mononuclear cells were isolated with Histopaque-1077 and treatments were applied to cells resuspended in RPMI-1640 at 500,000 cells per tube. Glyphosate was tested in duplicate at 1.25, 2.5, and 5 µM, but AMPA was not tested. The cells were then centrifuged, the supernatant discarded and 0.5 M sodium azide added. After centrifugation, the cell pellets were kept at −70 ºC until used for Western blot analysis. Two separate experiments were performed per test compound, with two donors each time. RIPA lysis buffer solution containing phosphatase and protease inhibitors was added to each cell pellet. The samples were then sonicated, incubated in ice, and centrifuged. The supernatant was recovered and 5 µL of each sample was placed in a 96-well plate for protein quantification. The Lowry assay was

performed with the DC Protein Assay kit and the concentration determined in a plate reader at 750 nm. The samples were then stored at −70 °C until use.
For the determination of phosphorylated Ku80 (phospho-T714) and Rad51 by Western blotting 35 µg of total protein was separated in a 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane with a Trans-Blot SD Semi-Dry Transfer Cell. After incubation with blocking solution and rinsing the appropriate membrane zones were isolated and incubated with rabbit anti-Rad51 polyclonal antibody or rabbit anti-phosphorylated Ku80 (phosphoT714) polyclonal antibody (p-Ku80). Incubation with primary goat anti-Actin polyclonal antibody was done for the determination of β-actin as the internal control. The membranes were then rinsed and incubated with secondary antibody goat anti-rabbit IgG-HRP for Rad51 and p-Ku80 detection and donkey anti-goat IgG-HRP for actin detection. After rinsing the protein fractions were quantified with a luminescence kit. Optical densities were measured with Quantity One software, version 4.1.1. The values obtained for each protein (Rad51 or p-Ku80) were normalized with respect to β-actin and the mean of the normalized negative controls. Results are presented as the % with respect to normalized negative controls. Two membranes were prepared per separate experiment with each test compound .

Statistical Analysis - Statistical calculations were made with the GraphPad Prism 6 software package and the results for γ-H2AX foci and optical density from Western blot analysis were evaluated with the Kruskal-Wallis test and Dunn's multiple comparison as a post hoc test. The value of etoposide as a positive control at the concentration of 50 µM was analyzed with the Mann-Whitney *U* test with respect to the negative control. Cytotoxicity was analyzed with linear regressions. The results were considered statistically significant if $p<0.05$.

Results
Identification of DNA double strand breaks (DSB) - For glyphosate, the mean ± SD of the percentage of cells with more than 10 γ-H2AX foci was 0.33 ± 0.52, 1.67 ± 1.37, 9.33 ± 7.94, 8.83 ± 4.36, and 3.17 ± 3.71 for 0, 0.4, 2, 10, and 50 µM (linear regression, $R^2 = 0.2$, $p = 0.02$). No effect was seen with AMPA. The positive control, etoposide, showed a significant correlation with dose ($R^2 = 0.82$, $p < 0.0001$) for cells with more than 10 foci. At 50 µM the positive control, etoposide, showed 89.67 ± 5.2% of cells with more than 10 γ-H2AX foci.

Cytotoxicity - The concentrations used for the determination of DSB were also used to assess the cytotoxicity of the test compounds that produced DSB. The survival range for glyphosate was 100% to 70% viability for concentrations ranging from 0.4 to 50 µM (p-value of 0.0047 for linear regression). Since no effects were seen on the formation of double strand breaks following exposure to AMPA, cell viability after treatment was not determined with this compound.

Quantification of p-Ku80 and Rad51 Proteins
The test compounds that showed positive for DSB were further tested to determine whether DNA recombination would be induced. Glyphosate was found to induce statistically significantly p-Ku80 protein in a dose-dependent manner for doses between 1.25 µM and 5 µM, whereas Rad51 was not significantly affected. Etoposide, i.e. the positive control, consistently induced p-Ku80, although with a wide variation between tests at 10 µM.

Discussion and Conclusions
Pesticides amongst which glyphosate were evaluated for their capacity to induce DNA double strand breaks, a lesion related to the formation of chromosomal rearrangements and leukemia risk. Glyphosate exhibited an ability to induce this kind of DNA damage in the form of phosphorylated γ-H2AX foci in the nuclei of human lymphocytes in at least two of the concentrations tested. AMPA tested negative. It is noticeable that the positive control, etoposide, induced more than 85 % of cells with more than 10 foci at 50 μM although it induced foci in a manner comparable to glyphosate at lower concentrations. This extent of DNA damage seems to be of relevance to the increase of repair proteins like p-Ku80, since glyphosate also produced a significant increase in this protein, whereas the rest of the test compounds that induced a lower percentage of cells with γ-H2AX foci did not. To evaluate proteins participating in DNA recombination, the highest concentration of glyphosate used was 5 µM to avoid cytotoxicity. Exposure of cells in a non-proliferative state to glyphosate not only induced the breakage of DNA, but also the phosphorylation of Ku80, a protein that participates in the c-NHEJ repair pathway. This pathway is known for being prone to error, introducing micro deletions or micro insertions, which could be mutagenic and alter cell behavior if they occur in coding or regulatory sequences. This is one possible outcome of the DSB in non-proliferating cells. Etoposide, the positive control, is well known as a topo II inhibitor capable of producing complex DSB. The DNA damage induced by etoposide in this study was comparable to the damage induced by paraoxon and glyphosate (DSB and pKu80 induction), so the question emerging from these results is whether the outcome for cells damaged by glyphosate would be similar to the outcome of cells damaged by etoposide and whether they would also induce chromosomal rearrangements.

Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to assess whether glyphosate and its metabolite AMPA produced DNA double strand breaks in human peripheral lymphocytes and whether proteins involved in DNA repair were induced. The results show that glyphosate, but not AMPA, increased the mean of the percent cells with more than 10 γ-H2AX foci, however, without a clear dose-effect relationship. Glyphosate was found to induce statistically significantly a protein involved in DNA repair, p-Ku80, at 5 µM without a dose-effect relationship (when measured as median OD).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Suárez -Larios *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	No guideline study for genotoxicity
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Only source reported: Sigma-Aldrich Mexico.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	Not applied
Test concentrations in physiologically acceptable range (< 1 mM)	Y	Up to 50 µM
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	SS increase at 2 concentrations but no dose effect relationship for γ-H2AX foci. SS increase of P-Ku80 at 5 µM but no dose-effect relationship in treated cultures (measured as median OD)
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	Results of this study are not corroborated by in vivo MN studies with much higher blood concentrations (approx. 300µM).
Not reliable		

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because glyphosate as a test chemical was not sufficiently characterized and the effect found on an indicator of DNA double strand breaks was not concentration related and occurred at concentrations that were much lower than the systemic concentrations (approx. 300 µM) of regulatory *in vivo* MN tests at 2000 mg/kg bw which were negative.

Assessment and conclusion by RMS:
It is agreed with the assessment and conclusion as presented by the applicant. In addition to the points mentioned by the applicant, the RMS notices that the positive results of double-strand breaks based on the surrogate marker γ-H2AX foci found in this public literature study are difficult to interpret since there is no guideline for this type of study and no information on validation of this assay in available. Further, the RMS notices that in this non-guidance compliant *in vitro* study, the formation of double strands breaks, cytotoxicity, and the investigation of two proteins involved in DNA repair was only investigated under conditions without metabolic activation, whereas usually conditions with and without metabolic activation should be studied *in vitro*. In addition, it is emphasized that the test compound is not identified completely. It would have been desirable to specifically state the purity of the test compound to verify that the observed effects are due to glyphosate and not due to impurities potentially present in the test item. In conclusion, the RMS therefore considered the study to be supportive.

B.6.4.4.10. *Genotoxicity – public literature, study 10*

Data point:	CA 5.4/010
Report author	Townsend, M. *et al.*
Report year	2017
Report title	Evaluation of various glyphosate concentrations on DNA damage in human Raji cells and its impact on cytotoxicity
Document No	doi.org/10.1016/j.yrtph.2017.02.002 E-ISSN: 1096-0295
Guidelines followed in study	None (although the comet assay itself was similarly conducted according to OECD 489)
Deviations from current test guideline	– Lysis conditions are a critical variable according to OECD 489. According to the guideline, slides should be immersed in chilled lysing solution for at least one hour (or overnight) at around 2-8 °C. In the study, it is only mentioned that cells were lysed for one hour, but without stating the temperature. The effect on the study outcome is unknown. – Number of cells/slides scored not in line with the OECD guideline (100 comets per time point and per treatment concentration cf. 150 cells per animal in at least 5 animals per dose).
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is not considered reliable for evaluation.

Full summary of the study according to OECD format
The DNA damage and cytotoxicity of various glyphosate concentrations on human cells was studied to evaluate DNA damaging potential. Utilizing human Raji cells, DNA damage was quantified using the comet assay, while cytotoxicity was further analyzed using MTT viability assays. Several glyphosate concentrations were assessed, ranging from 15 mM to 0.1 µM. It was found that glyphosate treatment is lethal to Raji cells at concentrations above 10 mM, yet has no cytotoxic effects at concentrations at or below 100 µM. Treatment concentrations of 1 mM and 5 mM induce statistically significant DNA damage to Raji cells following 30-60 min of treatment, however, cells show a slow recovery from initial damage and cell viability is unaffected after 2 h. At these same concentrations, cells treated with additional compound did not recover and maintained high levels of DNA damage. While the cytotoxicity of glyphosate appears to be minimal for physiologically relevant concentrations, the compound has a definitive cytotoxic nature in human cells at high concentrations.

Materials and Methods

Chemicals and reagents - Glyphosate (95 % w/w purity) was obtained from Sigma-Aldrich, Inc. (Milwaukee, WI).

Cell culture - Burkitt's Lymphoma (Raji) cells (ATCC CCL-86) were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Cells were cultured in RPMI 1640 and supplemented with 10 % FBS and 2 mM L-glutamine. Media were replaced every 48 hours and the cells used for testing were placed in exponential growth with a minimum viability of 95 %. Raji cells were selected for this study because the replication time is 18 hours allowing the assays to cover the entire cell cycle.

Cell viability assay - For use in the MTT viability assay, glyphosate was diluted in cell culture RPMI media to the final test concentrations and stored at 4 °C. Glyphosate stock solutions were diluted in RPMI media to their final test concentrations. Raji cells were incubated for 24 hours in a 96-well plate at 37° C and 5 % CO2. Afterwards, MTT reagent was added to each well. Following 3 hours of incubation, 100 µL of DMSO detergent was added to each well and incubated for 2 hours at 4 °C, and cytotoxicity evaluated at 570 nm absorbance.

Alkaline comet assay - Raji cells were incubated with either hydrogen peroxide as the positive control, PBS as the negative control, or glyphosate. The concentration and time points varied depending on the experimental run. The time intervals tested included 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 minutes. The concentrations of glyphosate used at each of the time points were 0.1, 1, 10, and 100 µM, and 1, 5, 10, and 15 mM. 200,000 cells per 100 µL were incubated with positive and negative controls and several concentrations of glyphosate at 37 ºC. Once treated, the cells were washed with PBS and suspended at 200,000 cells per 100 µL of PBS for the conduct of the comet assay. The test samples were mixed with low melting point agarose and layered on double frosted microscope slides and placed in alkaline lysis buffer for 60 minutes, rinsed with purified water and then placed in alkaline electrophoresis buffer for 20 minutes and submitted to electrophoresis (30 min, 24 V, 400 mA). After fixation and drying, the slides were stained with propidium iodide and imaged using a Zeiss Axioscope fluorescence microscope. All comets were scored using TriTek CometScore Freeware v1.5. Every experimental run tested a single concentration for multiple time points. Each time point contained a minimum of two slides as replicas. Approximately 50 comets were analysed per slide, totalling 100 comets per time point and per treatment concentration. Each concentration was replicated multiple times to ensure consistency. Comet assay results are reported as tail moment which is defined as the product of the tail length and the percentage of DNA in the tail. A similar protocol was followed to test the effects of secondary glyphosate exposure at 1 mM and 5 mM by adding 200 µL glyphosate solution to the cells after 60 minutes of initial treatment, while 200 µL of PBS was added to the negative control.

Statistical analysis - Relationships between exposure time and tail moment were modelled statistically using a natural spline to account for nonlinearity. The number of knots was selected based on Akaike Information Criteria (AIC) and parameters were estimated using least squares. P-values of <0.05 were considered statistically significant.

Results

MTT analysis of Raji cells exposed to various glyphosate concentrations for 24 hours indicated a significant loss of cell viability at 10 and 15 mM. Glyphosate concentrations of 5 mM and lower did not have a significant effect on viability when compared to the negative control.

Comet assay analysis of Raji cells exposed to 10 and 15 mM glyphosate indicated severe DNA damage and cell death soon after exposure. Within 30 min of treatment, all cells had adopted an apoptotic profile which is characterized by a loss of a defined comet head and a large, fragmented DNA tail. Tail moments were significant after just 10 minutes of glyphosate exposure. Raji cells exposed to glyphosate concentrations of 1 and 5 mM produced statistically significant DNA damage after 40 minutes of treatment. Tail moments reached a maximum after 60 and 80 minutes of treatment at 5 mM and 1 mM, respectively. A steady decrease in tail moment was observed in later time points and the cells were able to recover to full viability after 120 minutes of treatment. Cells exposed to 10 and 100 µM of glyphosate did not show statistically significant DNA damage, and the cells retained full viability throughout 120 minutes of treatment. Raji cells exposed to physiologically relevant concentrations of glyphosate (0.1-1 µM) for 120 minutes did not experience any significant DNA damage. Following treatment with 10 µM glyphosate, Raji cells showed no signs of DNA damage and the 'head' of the cells stayed intact throughout the 2-hour exposure time.

To further investigate the comet results at 1 and 5 mM, cells were treated again with glyphosate at these concentrations one hour after initial treatment. A significant difference could be observed between cells receiving

only primary treatment and cells receiving the additional treatment. Raji cells exposed twice to glyphosate did not show the same pattern of recovery, with tail moments reaching levels above 20 for 1 mM and 25 for 5 mM. Cells with only primary exposure to glyphosate showed a decrease in DNA damage, with tail moments dropping from 15 to 5.8 for 1 mM and 23.67 to 6.74 for 5 mM.

Discussion

The results show that the DNA damaging and cytotoxic potential of glyphosate is related to exposure length and treatment concentration. Glyphosate only induced significant DNA damage at concentrations several orders of magnitude larger than those attainable *in vivo*. The data support the established evidence that glyphosate is not genotoxic in human cells at physiologically relevant concentrations. While these data ultimately support glyphosate's classification as a potential carcinogen, they suggest that its effects are negligible when exposure is minimal. Furthermore, study results obtained at 1 mM and 5 mM suggest that cells initially damaged by glyphosate may have the ability to repair and regain viability after single exposure. The results show also that the extent of DNA damage changed drastically across different incubation time points. For example, incubation at 1 mM for one hour produced severe DNA damage whereas no DNA damage was evident after 2 hours at the same concentration. If cells had only been evaluated at this time point, results would suggest that there was no cytotoxic activity and the initial DNA damaging event would be missed. Cytotoxic activity might also be underestimated by standard viability assays in which the DNA damage is insufficient to induce cell death. Considering multiple time points made it possible to observe both DNA damage as well as the ensuing recovery.

Conclusion

Exposure of human cells to glyphosate produces minimal cytotoxicity and DNA damage at concentrations at or above 1 mM.

Assessment and conclusion

Assessment and conclusion by applicant:

The purpose of this study was to investigate the concentration and time dependent DNA damaging potential of glyphosate in Burkitt's B Cell Lymphoma (Raji) cells using the comet assay and MTT viability assay. The cells were exposed to glyphosate concentrations ranging from 0.1 µM to 15 mM and resulting DNA damage and loss of cell viability were measured after various lengths of exposure. DNA damage could only be observed at 1mM and higher which are concentrations that cannot be attained in vivo. The DNA damage seen at 1 and 5 mM reached its maximum between 60 and 80 minutes of incubation which returned to control values thereafter. To reach 1 mM of systemic concentration *in vivo* experimental animals have to be treated orally with glyphosate at dose levels that are much higher than those used in long term carcinogenicity studies which showed no carcinogenic effect of glyphosate.

This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for *in vitro* testing although no historical control data were reported. The significance for the risk assessment of glyphosate is limited because DNA damage has only been demonstrated at concentrations of glyphosate that cannot be attained in *in vivo* test systems.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Townsend *et al*., 2017	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95 %. Source: not reported.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	

Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)	Y/N	0.1 µM to 15 mM for alkaline comet assay
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Human cell exposure to glyphosate has minimal cytotoxicity and DNA damage at concentrations at or below 100 µM. Only effects found beyond 1 mM.
Overall assessment		
Reliable without restrictions	Y	
Reliable with restrictions		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria for in vitro testing although no historical control data were reported.		

Assessment and conclusion by RMS:

It is agreed with the applicant's study summary described above. We agree that the *in vitro* concentrations where cytogenicity was observed seem to be unreasonably high. It is, however, noted that although this study indicates that the DNA is repaired after treatment with glyphosate, this DNA might still contain induced mutations due to the fact that the repair mechanism for, e.g., double-strand breaks is not always error-free.

In contrast to the applicant, however, the study is considered supportive. Several reliability criteria required for a solid *in vitro* studies are not met. These included, but are not limited to the following: (i) the study seems to be conducted without a metabolic activation system only; (ii) although it is stated that positive controls were included in the assays, the results are not documented; (iii) no HCD are available (lab proficiency not proven). Furthermore, although the design of the comet assay itself is similar to OECD guideline 489, some deviations were noted (see above). Nevertheless, it is worth stating that the study authors consider glyphosate not genotoxic in human cells at physiologically relevant concentrations, which is in line with the available guideline- and GLP-compliant studies.

For the sake of completeness, some figures of the obtained results were included by the RMS below since the applicant did not include any tables or figures in its summary.

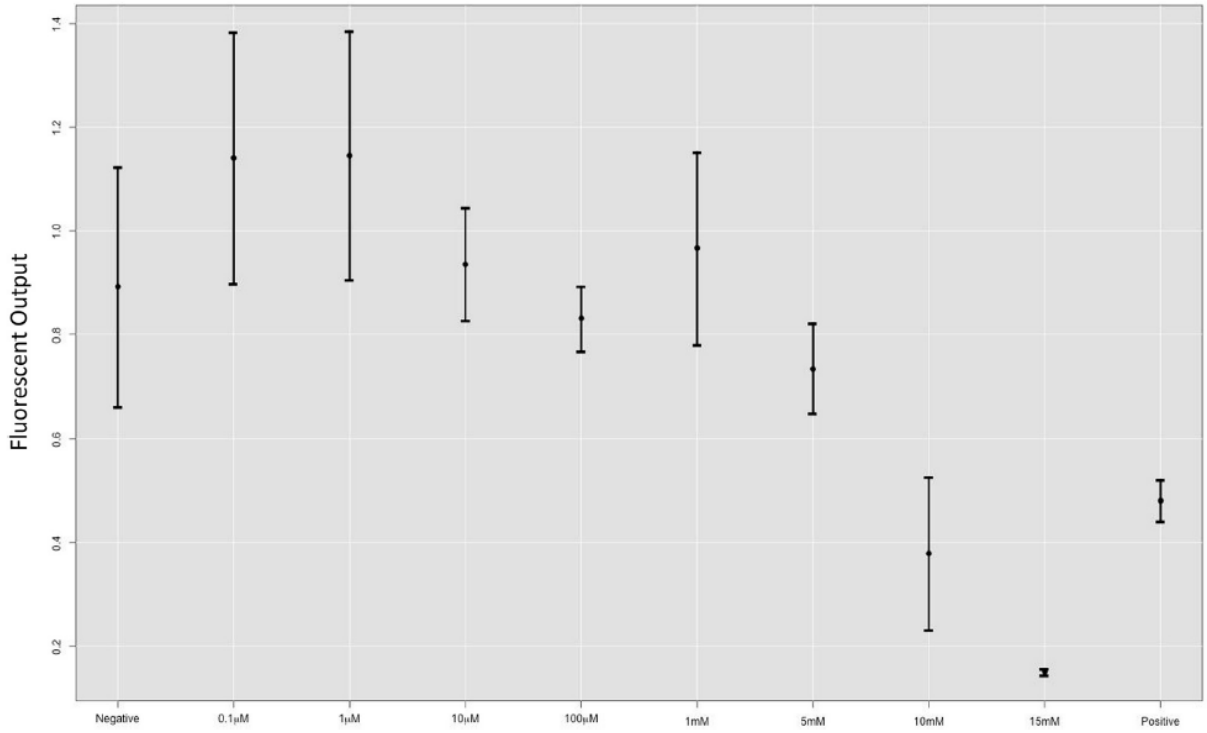


Figure B.6.4.4.10-1: MTT analysis of Raji cells exposed to various glyphosate concentrations. Hydrogen Peroxide (positive) was utilized as a control to measure thorough cell death, and cells suspended in cell growth media (negative) was utilized as a control for standard cell death as a result of treatment conditions. Following 24 h of incubation with glyphosate, there was a significant loss of cell viability following treatment with 10 mM and 15 mM glyphosate. Concentrations of 5 mM and lower did not have a significant loss of viability when compared to the negative control. This indicates the damage to Raji cells at 15 mM and 10 mM glyphosate was enough to sustain complete cell death, while concentrations at or below 5 mM sustained cell viability.

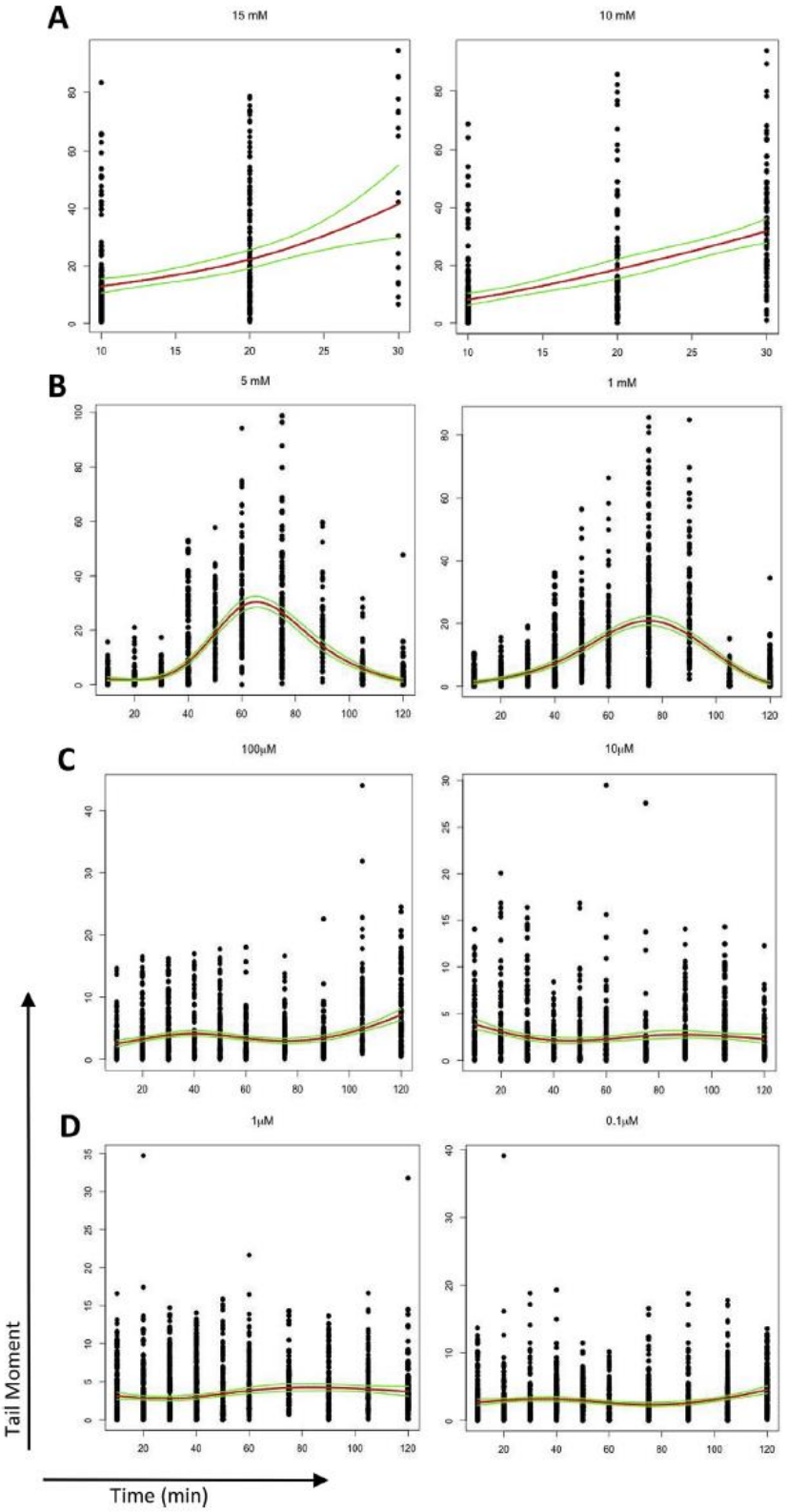


Figure B.6.4.4.10-2: Tail Moment values of cells treated with various concentrations of glyphosate across 2 h of treatment. Tail moment values (quantifiable measure of DNA damage) are listed on the y-axis (scale varies at different concentrations), and treatment times are labeled on the x-axis. Each concentration was individually evaluated and assigned a 95% confidence interval, which is displayed in green, while the mean value is shown in red. **A,** Cells exposed to glyphosate concentrations of 15 mM and 10 mM sustained severe DNA damage with tail moments above 25, which was indicative of cell death. Within 30 min of treatment, all cells had adopted the

characteristic profile of a dead cell. **B**, Raji cells exposed to glyphosate concentrations of 5 mM and 1 mM had statistically significant DNA damage after 60-75 min of treatment. This damage was not present in later time points and cells were able to recover full viability after 120 min of treatment. **C,** Cells treated with 100 mM and 10 mM of glyphosate did not show statistically significant DNA damage, and cells retained full viability throughout the full 120-min treatment. **D,** Physiologically relevant concentrations of glyphosate were exposed to Raji cells over a 120 min period and did not experience any significant DNA damage.

B.6.4.4.11. *Genotoxicity – public literature, study 11*

Data point:	CA 5.4/011
Report author	Roustan, A. *et al.*
Report year	2014
Report title	Genotoxicity of mixtures of glyphosate and atrazine and their environmental transformation products before and after photoactivation
Document No	E-ISSN: 1879-1298
Guidelines followed in study	Micronucleus assay similar to OECD Test Guideline 487
Deviations from current test guideline (OECD 487 (2016))	– OECD GL 487 requires cells to be exposed for 3-6 h with and without metabolic activation, and sampled after 1.5-2.0 cell cycles lengths after beginning of exposure; and additionally, cells should continuously be exposed for 1.5-2.0 cell cycle lengths without metabolic activation. In the current study, however, cells were only exposed for 3 hours. – Test chemicals not sufficiently characterised. Test substance stability and test concentration not analytically verified. – No positive control and no HCD included.
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive

Full summary of the study according to OECD format

The photo-inducible cytogenetic toxicity of glyphosate, atrazine, aminomethyl phosphoric acid (AMPA), desethyl-atrazine (DEA), and their various mixtures was assessed by the in vitro micronucleus assay on CHO-K1 cells. Results demonstrated that the cytogenetic potentials of pesticides greatly depended on their physico-chemical environment. The mixture made with the four pesticides exhibited the most potent cytogenetic toxicity, which was 20-fold higher than those of the most active compound AMPA, and 100-fold increased after light-irradiation. Intracellular ROS assessment suggested the involvement of oxidative stress in the genotoxic impact of pesticides and pesticide mixtures.

The RMS notes that the summary presented below is focussed on glyphosate and AMPA, and not on the various mixtures of different substances since these are not considered relevant in the frame of the current application for renewal.

Materials and methods

Chemicals - Glyphosate and AMPA were purchased from Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.

Cell culture - Experiments were performed in CHO-K1 cells (ATCC-LGC Standards Sarl, Molsheim, France). CHO-K1 cells were maintained in McCoy's 5A medium supplemented with 10 % bovine calf serum, 1 mM glutamine, and 100 U/mL-10 µg/mL penicillin-streptomycin. Cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2.

Metabolic activation mixture (S9-mix) - The metabolic activation system used was a 9000g centrifuged supernatant (S9) of a 10 % liver homogenate prepared from male Sprague-Dawley rats treated with a single injection of Aroclor 1254 (500 mg/kg bw), 5 days before sacrifice. The protein concentration in the S9 homogenate was 26 mg/mL.

For the micronucleus assay, the S9-mix contained 10 % S9, 5 mM glucose-6-phosphate, 4 mM NADP, 33 mM KCl and 8 mM $MgCl_2$ diluted in PBS.

Photoactivation - To reproduce photoinduction during exposure to glyphosate and AMPA in the environment, the micronucleus assay was performed with a photoactivation procedure. Irradiation of cell cultures was carried out 1 hour after the addition of test compound using a solar simulator Suntest CPS+ apparatus equipped with a xenon arc lamp (1,100 W), a special glass filter restricting transmission of light below 290 nm and a near IR-blocking filter. The irradiance for the photoactivation was fixed at 750 W/m^2 throughout testing. The combined light dose was 4.5 J/cm^2 for one minute irradiation (0.03 J/cm^2 of UVB, 0.41 J/cm^2 of UVA and 4.06 J/cm^2 of visible light). This irradiation dose is representative of a 1-3 minute period of solar exposure during a clear summer day in the United Kingdom. The temperature of the samples was kept at 4 °C. UVA-visible light (320-800 nm) was obtained using the solar ID65 filter plus a window glass filter.

Micronucleus assay - A total of 50,000 CHO-K1 cells was plated in chamber slides and incubated for 24 hours at 37 °C in a humidified atmosphere containing 5 % CO_2. Various concentrations of glyphosate (5, 10, 50, and 100 µg/mL) and AMPA (0.005, 0.01, 0.05, and 0.1 µg/mL without S9-mix; 0.1, 0.5, 1, and 5 µg/mL with S9-mix; 0.00005, 0.0001, 0.0005, and 0.001 µg/mL with light irradiation) were incorporated into duplicate CHO-K1 cell cultures. To determine the background DNA-damage levels in CHO cells in the dark and with light irradiation, 2 negative controls were added for glyphosate and AMPA: culture medium and PBS. Mitomycine C (0.06 µg/mL) without S9-mix and benzo[a]pyrene (5 µg/mL) with S9-mix were selected as positive controls. After 3 hours of exposure, cells were rinsed with PBS and incubated in fresh medium containing cytochalasin B (3 µg/mL) for an additional 24 hours to stop cytokinesis. At the end of the incubation period, cells were rinsed twice with PBS and fixed with methanol. The slides were air dried and stained with 5 % Giemsa stain in Milli-Q water for 15 minutes. The Cytokinesis Blocked Proliferation Index (CBPI) was used to select adequate concentrations for the assessment of micronuclei. CBPI was determined by scoring the number of mononucleated (M1), binucleated (M2), and trinucleated (M3) cells among 500 Giemsa-stained cells with well-preserved cytoplasm: CBPI = [(M1) + (2 x M2) + (3 x M3)]/500. When a cytotoxic effect was observed, micronucleated cell rates were determined for concentrations inducing less than a 50 % decrease of CBPI. When no cytotoxic effect was observed, the maximal concentration assessed was 100 µg/mL. When a clastogenic/aneugenic activity was observed, 4 relevant concentrations were selected to obtain a dose-response relationship. A total of 2000 binucleated cells were examined for each concentration, and micronuclei were identified according to the morphological criteria previously defined.

Intracellular ROS analysis - Intracellular ROS was determined with the cell-based OxiSelect™ Intracellular ROS Assay Kit using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). After diffusion into the cell, this fluorescent probe is deacetylated by cellular esterases to non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH), which is rapidly oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by ROS. A total of 50,000 cells were seeded in a black 96-well cell culture plate and incubated at 37 °C for 24 hours. Cells were rinsed 3 times with PBS and incubated in 100 µL of DCFH-DA/media solution at 37 °C for 60 minutes. Cells were subsequently rinsed with PBS and treated with glyphosate or AMPA. Experiments were performed in triplicate. The concentrations tested were the same as those assessed for the micronucleus assay. H_2O_2 (100 µM) was used as the positive control. After a 30-minute incubation period, fluorescence was measured with a fluorometric plate reader at 480 nm and 530 nm.

Statistical analysis – Statistically significant differences between negative controls and treated samples were determined using the ^{2}test. The standard micronucleus assay was considered positive when a dose-response relationship could be established between the numbers of micronucleated cells and the concentrations of pesticide solutions, and when at least one concentration induced a significant increase of micronucleated cells as compared to the medium-only control culture. The dose-response relationships were calculated by nonlinear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Minimal Clastogenic Concentration (MCC) was defined as the lowest concentration of test item (µg/mL) that induced a significant increase of micronucleated cells. The Cytogenetic Potency (CP) was defined as the slope of the dose–response curves. It was calculated by non-linear regression analysis with TableCurve2D®.
For the determination of ROS, the dose-response relationships were calculated by linear regression analysis with TableCurve2D®. Model significance was based on three criteria: (i) correlation coefficient r^2 being > 0.62; (ii) model probability (P) being < 0.05; (iii) error probability (PE) being > 0.05. The Oxidative Potency was defined as the slope of the dose-response curves and was calculated according to a standard curve obtained with various concentrations of fluorescent DCF.

Results
No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate in the dark and without S9-mix at concentrations up to 100 µg/mL. In the presence of S9-mix a statistically significant and dose-related increase was noted from 10 µg/mL. The calculated minimal clastogenic concentration (MCC) was 5.8 µg/mL. With light irradiation a statistically significant increase in BMC was noted at the highest concentration of glyphosate tested (100 µg/mL) with an MCC of 93.4 µg/mL.
Without S9-mix a statistically significant and dose-related increase in BMC was produced by AMPA from a concentration of 0.01 µg/mL with an MCC of 0.006 µg/mL. In the presence of S9-mix a statistically significant increase in BMC was seen with AMPA from 1 µg/mL with an MCC of 0.78 µg/mL. With light irradiation, the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL with an MCC of 0.0004 µg/mL.
The oxidative potency of H_2O_2, used as a positive control, was 333.3 nM DCF/µM^{-1} (data not shown). Only AMPA exerted an elevated oxidative effect (5.9 nM DCF/µg mL^{-1}), whereas the oxidative potency of glyphosate was very low.

Discussion
The results obtained in the present study confirmed the cytogenetic toxicity of glyphosate and AMPA *in vitro*. In the dark, glyphosate was not directly active in the absence of S9-mix, but it induced micronuclei in the presence of S9-mix. On the contrary, without S9-mix, AMPA displayed a direct cytogenetic effect which was 1,000 fold higher than that of its parent compound. The weak mutagenic activity of glyphosate with S9-mix could be explained by its weak metabolization yield *in vivo*. After sunlight irradiation, glyphosate was weakly active, whereas the cytogenetic effect of AMPA was about 20-fold increased. Since the photodegradation of glyphosate by UV light has been shown to produce AMPA, the photoinducible genotoxic potential of glyphosate could be partially explained by the formation of its metabolite AMPA in the intracellular environment. In the present study, cells were subjected to a very low irradiation that did not induce intrinsic DNA damage. The results of the present study implied more probably the involvement of a photoinduced oxidative stress, which transformed glyphosate and AMPA into photoactivated intermediates favouring interactions with cellular targets.

Conclusion
The results of this study demonstrated that light-irradiation, corresponding to a few minutes of solar exposure, greatly potentiated the cytogenetic impact of AMPA. *In vitro* experiments showed that the genotoxic impact of pesticides greatly depend on their physico-chemical environment,

Assessment and conclusion

Assessment and conclusion by applicant:
The cytogenetic effect of two herbicides (glyphosate and atrazine), their metabolites (AMPA and DEA), and mixtures thereof was investigated in CHO-K1 cells in the *in vitro* micronucleus test. Only the results of glyphosate and AMPA tested alone are reported and discussed in the summary. Glyphosate and AMPA were tested with and without metabolic activation and with light irradiation. Also the potency of glyphosate and AMPA to produce ROS was investigated. The concentrations tested ranged from 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA. No statistically significant increase in the incidence of bi-micronucleated cells (BMC) was observed with glyphosate at concentrations up to 100 ug/mL in the dark and without metabolic activation. However, a statistically significant and dose-related increase in BMC was noted from 10 µg/mL in the presence of metabolic activation. With light irradiation a statistically significant increase in BMC was noted for glyphosate at a concentration of 100 µg/mL. AMPA produced a statistically significant and dose-related increase in BMC from a concentration of 0.01 µg/mL in the dark and without metabolic activation. With metabolic activation a statistically significant increase in BMC was seen with AMPA from 1 µg/mL. With light irradiation the lowest test concentration of AMPA with a statistically significant increase in BMC was 0.0005 µg/mL. Only AMPA was found to produce an elevated oxidative effect, whereas the oxidative potency of glyphosate was very low. The results of glyphosate in the *in vitro* micronucleus test with metabolic activation reported in this study are surprising since glyphosate is essentially unmetabolized *in vitro* in the presence of a rat liver S9 homogenate. Moreover, these results are not corroborated by regulatory *in vivo* micronucleus tests in the mouse dosed up to more than 2,000 mg/kg bw.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Roustan *et al*., 2014	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	In vitro MN test compliant with OECD TG 487.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity not reported. Source: Sigma-Aldrich Chemical Company, St Quentin-Fallavier, France.
Only glyphosate acid or one of its salts is the tested substance	N	Other pesticides (atrazine, desethyl atrazine (DEA)) were tested and mixtures thereof.
AMPA is the tested substance	Y	Tested alone and as mixtures with glyphosate, atrazine and DEA.
Test system clearly and completely described	Y	CHO-K1 cells.
Test conditions clearly and completely described	Y	CHO-K1 cell MN test.
Metabolic activation system clearly and completely described	Y	S9-mix.
Test concentrations in physiologically acceptable range (< 1 mM)	Y	From 5 to 100 µg/mL for glyphosate and from 0.00005 to 5 µg/mL for AMPA.
Cytotoxicity tests reported	Y	Cytokinesis Blocked Proliferation Index (CBPI) and incidence of bi-micronucleated cells (BMC).
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate and AMPA tested were not sufficiently characterized and no positive and control historical data were reported. The *in vitro* micronucleus test carried out was in compliance with OECD TG 487.		

Assessment and conclusion by RMS:

It is agreed with the applicant's summary of the study. In contrast to the applicant, the deviations from the OECD guideline (see above) and the noted shortcomings of the study lead to the conclusion that the study is considered supportive only.

As already pointed out by the applicant, cytogenicity was only observed in tests with metabolic activation, but not without. These results seem a bit odd since glyphosate is essentially unmetabolized *in vitro* in the presence of a rat liver S9 homogenate. However, as the purity of the test substance is not reported, it is not possible to exclude any effect from impurities present in this specific source which might be activated by the rat liver S9 homogenate.

B.6.4.4.12. *Genotoxicity – public literature, study 12*

Data point:	CA 5.4/012
Report author	Mañas, F. *et al.*
Report year	2013
Report title	Oxidative stress and comet assay in tissues of mice administered glyphosate and AMPA in drinking water for 14 days
Document No	ISSN: 1666-0390
Guidelines followed in study	None (although the comet assay shows some similarity with OECD GL 489).
Deviations from current test guideline	– Sex of the animals is not reported. – Number of dose levels not in line with OECD GL 489 (at least three dose levels) – Number of scored nucleoids not in line with OECD GL 489. – No positive controls and HCD available (lab proficiency not proven). – The description of the assay is very limited. Important details are missing. – Although not part of OECD GL 489, it is noted that also the description of the method for the determination of oxidative stress parameters is very limited.
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is considered supportive

Full summary of the study according to OECD format

In this study the authors determined the levels of thiobarbituric acid reactive substances (TBARs); quantified superoxide dismutase (SOD) and catalase (CAT) activity in liver, kidney, lung and heart, and performed the comet assay in blood and liver of mice administered glyphosate (40 or 400 mg/kg bw/day) or AMPA (100 mg/kg bw/day) in drinking water for 14 days. Exposure to glyphosate 400 mg/kg bw/day induced a statistically significant ($p < 0.05$) decrease of SOD activity in heart and an increase in CAT activity in kidney. In the comet assay there were statistically significant differences in all the treatments and tissues studied in comparison to control animals ($p \leq 0.01$). The major results of this study were that mice administered glyphosate or AMPA in drinking water for 14 days induced a significant increase in DNA damage in liver and blood but minor effects on oxidative stress parameters. DNA effects on liver and blood indicate that these compounds could be of concern in terms of their potential to damage the genetic material, and that oxidative stress does not seem to be the mechanism causing that effect.

Materials and Methods

Chemicals - Analytical grade glyphosate (96 % purity), and analytical grade AMPA, (99 % purity) were obtained from Sigma-Aldrich, Argentina.

Animals - Twenty-four Balb C mice of approximately 45 days of age were used in this study. Animals were obtained from the animal facility of the Faculty of Agronomy and Veterinary Medicine, National University of Río Cuarto. Four groups of six animals each (a control group, 2 groups treated with glyphosate and a group treated with AMPA) were used for the determination of oxidative stress (TBARs, SOD and CAT) and DNA damage in the comet assay. During 7 days prior to the beginning of the study, the volume of water ingested per day was measured in every 2 animals per cage to determine the concentration of glyphosate and AMPA in the drinking water to be supplied to each group. Mice received approximately 40 or 400 mg/kg bw/day of glyphosate and 100 mg/kg bw/day of AMPA via drinking water. The control group received drinking water without test compound. After 14 days of exposure, before sacrifice, peripheral blood was drawn from the tail vein to perform the comet assay. At necropsy, heart, lungs, liver, and kidneys were removed for the determination of TBARs, SOD and CAT and frozen at -80 ºC pending analysis. A part of the liver was homogenized in phosphate buffer (pH 7.4) immediately after excision for the conduct of the comet assay. All determinations were performed in triplicate.

Single cell gel electrophoresis assay in mouse blood and liver - The protocol followed the general guidelines proposed by Singh et al. (Exp. Cell Res., 175, 184-191, 1988) with minor modifications. The slides were fixed in absolute ethanol, stained with ethidium bromide and scored using fluorescence microscopy. Images of 100 "nucleoids" counted for each animal were captured with a camera attached to the fluorescence microscope and linked to the Comet Score 1.5 software. Tail moment (TM), percentage of DNA in tail (% of DNA) and tail length (TL) were used to estimate DNA damage (in arbitrary units).

TBARs, SOD and CAT determinations - Tissue homogenates (10 %) were prepared in chilled 0.05 M potassium phosphate buffer at pH 7.4. TBARs concentrations, expressed as nmol of malondialdehyde (MDA)/g of tissue, were measured spectrophotometrically at 532 nm in liver and kidney homogenates. TBARs concentrations were determined using a standard curve at different concentrations of MDA versus optical density, individually prepared for each tissue. Superoxide dismutase (SOD) activity was assessed spectrophotometrically in the supernatant of liver homogenates. One unit of enzymatic activity has been defined as the amount of enzyme capable of causing 50 % inhibition of auto-oxidation of epinephrine. Catalase (CAT) activity was measured at 240 nm by the decomposition of the H_2O_2.

Statistical analysis
Statistical analysis was performed using Prism software (PRISM, 1997). The Kolmogorov-Smirnov test was performed to verify if the results follow a normal distribution. ANOVA followed by the Dunnett's test, or the Kruskal-Wallis test followed by the Dunn's test were performed for data with and without normal distribution, respectively.

Results
The volume of water ingested per animal/day did not show any statistically significant differences among the experimental groups throughout treatment. No statistically significant differences were found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD in the heart and an increase in CAT in the kidney at a daily glyphosate dose of 400 mg/kg bw. There were no statistically significant changes in the concentrations of MDA/g of tissue at 40 and 400 mg/kg bw/day of glyphosate and at 100 mg/kg bw/day of AMPA.
Tail intensity, tail length and tail moment were statistically significantly elevated in blood and liver of all dosed groups (glyphosate and AMPA) with the exception of tail intensity in liver at 40 mg/kg bw/day glyphosate.

Discussion and Conclusion
The presence of glyphosate or AMPA in drinking water did not affect water consumption. Statistically significant differences in oxidative stress (decrease in SOD activity in the heart and increase in CAT activity in the kidney) were only observed in mice treated with glyphosate at 400 mg/kg bw for 14 days. A non-statistically significant decrease in SOD activity was observed in all tissues of animals treated with 100 mg/kg bw AMPA but no effect of AMPA was found on CAT activity. Liver was the only tissue where no effects were recorded, neither in TBA reactive substances nor in SOD and CAT activity.
In the comet assay of blood statistically significant differences from control were seen for glyphosate and AMPA in tail intensity, tail length and tail moment. Tail moment values in the comet assay were similar for both glyphosate and AMPA treatments. In this study, genotoxic changes were observed with glyphosate at 40 and 400 mg/kg bw/day and AMPA at 100 mg/kg bw/day. However, statistically significant changes in the levels of SOD and CAT especially in heart and kidney were only seen with glyphosate at 400 mg/kg bw/day. This suggests that the genotoxic effects of glyphosate and AMPA are much more important than the indicators of oxidative stress.

Assessment and conclusion

Assessment and conclusion by applicant:
The objective of this study was to investigate the effect of glyphosate and AMPA on indicators of oxidative stress and DNA integrity in mice after oral exposure for 14 days via the drinking water. The results of this study indicate that no statistically significant differences have been found in liver, kidney, lung and heart for all oxidative stress parameters measured with the exception of a decrease in SOD activity in the heart and an increase in CAT activity in the kidney at a daily glyphosate dose of 400 mg/kg bw. There was an increase in CAT activity in the lung but this was not statistically significant and did not show a dose-effect relationship. A statistically significant increase in DNA damage parameters was observed for glyphosate and AMPA with the exception of tail intensity in the liver for glyphosate at 40 mg/kg bw/day. No clear dose-effect relationship

was evident for DNA damage parameters in blood after treatment with glyphosate. A dose-effect relationship was present for tail length and tail moment in the liver.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory *in vivo* MN tests in the mouse with negative results.

Reliability criteria for *in vivo* toxicology studies made by the applicant

Publication: Manas et al., 2013	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	2 dose groups for glyphosate, one dose group for AMPA
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Very old determination methods applied
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 96 %. Source: Sigma-Aldrich, Argentina.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	Y	Purity of 99 %. Source: Sigma-Aldrich, Argentina.
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Exposure via drinking water
Dose levels reported	Y	
Number of animals used per dose level reported	Y	
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported		Only 2 dose levels used. DNA damage (comet) at 40 and 400 mg/kg bw/day (no dose-effect relationship) not confirmed by regulatory genotoxicity (MN) studies up to 2000 mg/kg bw/day
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the increased DNA damage seen (only 2 dose levels tested for glyphosate with too few animals) didn't show a dose-effect relationship in blood and occurred at dose levels (40 and 400 mg/kg bw/day) that are much lower than the 2000 mg/kg bw used in regulatory in vivo MN tests in the mouse with negative results.		

Assessment and conclusion by RMS:
It is agreed with the study summary as presented by the applicant. In contrast to the applicant, however, the study is considered as supportive only due to the deviations noted above.

As there is no dose-response observed for glyphosate and as there are no historical control or positive control data provided for this laboratory, the results are difficult to interpret. Together with the limitations of the study reported above the summary, this study does not give clear evidence for positive results for glyphosate or AMPA.

For the sake of completeness, some results obtained in the study were included by the RMS below since the applicant did not include any tables or figures in its summary.

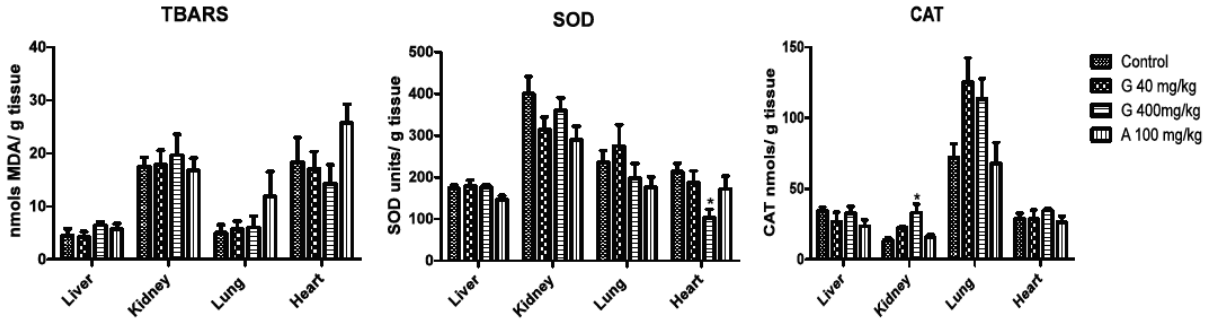


Figure B.6.4.4.12-1: Changes in the levels of TBARs, SOD and CAT activity in liver, kidney, lung and heart in mice administered Glyphosate or AMPA in drinking water during 14 days. n= 6 animals per group. p<0.01 respect to the control, Dunn test. G: glyphosate, A: AMPA. Each determination was performed by triplicate.

Table B.6.4.4.12-1: Comet assay in blood and liver of mice administered Glyphosate or AMPA in drinking water during 14 days. n= 6 animals per group. *p< 0.05, **p< 0.001, ***p< 0.0001 respect to the control, Dunn test.

	Tail intensity mean ± SEM (arbitrary units)		Tail length mean ± SEM (arbitrary units)		Tail moment mean ± SEM (arbitrary units)	
	blood	liver	blood	liver	blood	liver
Control	± 1.73 0.85	± 1.04 0.82	± 7.02 6.48	± 2.91 1.09	± 2.98 1.08	± 7.14 3.41
14 days Glyphosate 40 mg/Kg	± 3.39*** 1.55	± 1.21 0.91	16.60*** ± 0.99	± 3.97** 3.25	± 8.54*** 7.82	± 7.92* 3.99
14 days Glyphosate 400 mg/Kg	± 3.64*** 1,17	± 1.62*** 0.71	16.68*** ± 0.69	± 9.36*** 4.92	± 9.06*** 5.15	20.59*** ± 15.47
14 days AMPA 100 mg/Kg	± 2.86*** 1,36	± 1.28*** 0.53	14.70*** ± 0.95	± 7.02*** 4.03	± 8.45*** 6.43	14.99*** ± 9.09

B.6.4.4.13. *Genotoxicity – public literature, study 13*

Data point:	CA 5.4/013
Report author	Koller, V. J. *et al.*

Report year	2012
Report title	Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells
Document No	DOI 10.1007/s00204-012-0804-8 E-ISSN: 1432-0738
Guidelines followed in study	SCGE (single cell gel electrophoresis; comet assay) assays were performed according to the guidelines described by Tice et al. (2000) and the CBMN Cytome assay according to Fenech (2007). The corresponding Guidance Documents are OECD 489 and OECD 487
Deviations from current test guideline	– Cell type (buccal cells) – Treatment schedule not in line with the guidelines (20 min only instead of 3-6 h and continuous treatment for the MN assay, and 2-6 and 16-26 h for the comet assay (although it is recognised that this is applicable for *in vivo* comet assays)). – Only conditions without the addition of a metabolic activation system were investigated. – No positive control included for the comet assay. HCD is available for the comet assay and the MN assay, however, these data are based on 3 and 4 independent experiments only. – Although not described in OECD GL 487 or 489, it is noted that the description of the different methods for the determination of cytotoxicity is very limited.
GLP/Officially recognised testing facilities	No, not conducted under GLP/ Officially recognised testing facilities.
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG:** The study is not considered reliable due to the deviations noted above (supportive only).

Full summary of the study according to OECD format

Aim of this study was to investigate the cytotoxic and genotoxic properties of glyphosate (G) and Roundup (R) (UltraMax) in a buccal epithelial cell line (TR146), as workers are exposed via inhalation to the herbicide. R induced acute cytotoxic effects at concentrations > 40 mg/L after 20 min, which were due to membrane damage and impairment of mitochondrial functions. With G, increased release of extracellular lactate dehydrogenase indicative for membrane damage was observed at doses > 80 mg/L. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses > 20 mg/L. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20 min exposure to 10 -20 mg/L, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/L). R was under all conditions more active than its active principle (G).

Materials and Methods

Chemicals - Glyphosate (purity 95 % w/w) and Roundup (Roundup Ultra Max, 450 g/L glyphosate acid) were obtained from Monsanto Europe S.A.

Storage and cultivation of the indicator cells - The human cell line TR146 was cultured under standard conditions in DMEM supplemented with 10 % heat-inactivated fetal calf serum. The medium was changed every 2–3 days. When the cultures reached confluency, the cells were washed with Dulbecco's PBS, detached with TrypLE Express, centrifuged, and sub-cultured. TR146 cells express ultrastructural characteristics of normal human buccal epithelial cells, e.g. intermediate filaments, microvilli-like processes, and lack of complete keratinization.

Cytotoxicity assays - For cytotoxicity experiments, $5x10^4$ indicator cells were seeded in 96-well plates and exposed to concentrations of glyphosate and Roundup ranging from 0 to 200 mg glyphosate equivalents/L for 20 minutes. Toxicity due to damage of the cell membrane was determined with the extracellular LDHe assay that is based on the measurement of the oxidation of NADH to NAD. Alterations of mitochondrial functions were studied in XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide) assays that measure succinate dehydrogenase activity of viable cells. The SRB test was used to monitor total protein synthesis as a marker of cell proliferation. SRB binds to cellular proteins and can be quantified after solubilization. The neutral red assay was conducted to evaluate membrane integrity and lysosomal activity of the cells. All

experiments are based on spectrophotometric measurements and were evaluated with an automated microplate reader. In all experiments, three measurements were performed per dose and repeated at least once.

Single-cell gel electrophoresis (SCGE) assay - SCGE assays were performed to determine the effect of glyphosate and Roundup on DNA stability. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 2000 mg glyphosate equivalents/L. TR146 cells were seeded in 24-well plates and allowed to attach. Thereafter, the culture medium was replaced by 400 µL of different concentrations of glyphosate dissolved in serum-free medium. After incubation in the dark at 37 ºC and 5 % CO_2 for 20 minutes, the medium was discarded and the cells washed twice with DPBS and detached. After two washing steps with DMEM and centrifugation, the pellets were resuspended in low melting point agarose, spread onto slides precoated with normal melting point agarose and lysed in the dark at 4 ºC for at least 1 hour. After 20 minutes unwinding in an alkaline electrophoresis solution electrophoresis was carried out. Air-dried slides were stained with ethidium bromide and the percentage of tail DNA was measured using a computer-aided image analysis system (Comet IV, Perceptive Instruments Ltd., Haverhill, UK). For each experimental point, 3 cultures were made in parallel and from each culture, one slide was prepared and 50 randomly distributed cells evaluated.

Cytokinesis-block micronucleus (CBMN) cytome assay - The CBMN assay using the cytochalasin B technique was performed to determine the effect of glyphosate and Roundup on chromosome integrity. The concentrations of glyphosate and Roundup used in this assay ranged from 0 to 20 mg glyphosate eq./L. Into 6-well plates, 4.5 x 10^5 cells were seeded and allowed to attach overnight. After treatment for 20 minutes with the test compounds or 100 µg/mL methyl methanesulfonate as the positive control, the cells were washed twice with PBS and cultured in DMEM containing 10 % FCS and cytochalasin B for 48 hours. Subsequently, the cells were washed twice with DPBS and harvested. The slides were made by cytocentrifugation and subsequently air-dried, fixed, and stained with Diff Quick stain. The total number of micronuclei (MNi) in binucleated cells (BN) as well as the number of binucleated cells with micronuclei (BN-MNi), nuclear buds (NB), nucleoplasmic bridges (NPB), and apoptotic and necrotic cells was determined. Cells that divided after addition of cytochalasin B were recognized as binucleated. The nuclear division index (NDI) was determined in 500 cells. For each experimental point, TR146 cultures were prepared in triplicate. From each culture, >1,000 binucleated cells were evaluated under 400-fold magnification (Nikon Photophot-FXA, Tokyo, Japan).

Statistical analysis - Data analyses of the cytotoxicity and SCGE assays were performed with the GraphPad Prism 5 Project software system. Results are reported as means ± standard deviations (SD). The results were analyzed using one-way ANOVA and Dunnett's test and p values ≤0.05 were considered as statistically significant. The chi-square test with Yate's correction was used for the evaluation of the CBMN experiments and p values ≤0.05 were considered statistically significant.

Results

Cytotoxicity - Clear differences have been found between the effects of glyphosate and Roundup. Roundup was in all cases more cytotoxic than the active ingredient. In the LDHe test Roundup NADH consumption was statistically significantly increased from 10 mg glyphosate eq./L on whereas this was the case with glyphosate from 80 mg/L. In the XTT test, a statistically significant decrease in cellular integrity was seen with Roundup from concentrations of 40 mg glyphosate eq./L on while there was no effect with glyphosate up to 200 mg/L. In the SRB test there was a statistically significant decrease in cellular integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate up to 200 mg/L. The same difference in cytotoxicity was evident in the NR assay where Roundup produced a statistically significant decrease in cell integrity from 100 mg glyphosate eq./L on whereas there was no effect with glyphosate. The LC_{50} values for the cytotoxicity of Roundup were about 100 mg glyphosate eq./L for the XXT test, 140 mg/L for the NR assay and 150 mg/L for the SRB assay.

SCGE assay - In the SCGE assay, glyphosate produced at statistically significant increase in tail intensity from 20 mg/L on without a dose-effect relationship from 40 to 2,000 mg/L. Cell integrity following exposure to glyphosate remained constant around 100% for all doses. A statistically significant and dose dependent increase in tail intensity was observed with Roundup from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L, a dose level with 0 % cell integrity. Therefore, it can be not excluded that cytotoxic effects caused by high concentrations may have led to differential results in the SCGE assays.

CBMN assay - The endpoints recorded in this assay were the frequency of binucleated (BN) cells with micronuclei (MNi), the total number of MNi, nuclear buds (NB) and nucleoplasmic bridges (NPB). After addition of cytochalasin B for 48 h, more than 75 % of the cells were binucleated (BN) and the nuclear division index (NDI) in untreated cultures was 1.89 ± 0.09. Treatment of the cells with 100 µg/mL of the positive control MMS for 20

minutes produced a significant induction of BN cells with MNi, total number of MNi, NB and NPB. Exposure of the cells to glyphosate and Roundup at 10, 15 and 20 mg glyphosate eq./L for 20 minutes led to a statistically significant and dose-dependent increase of the frequency of MN, BN-MN, and NB. In the case of the NPB, the only significant effect was obtained with the highest dose of Roundup (20 mg/L); while with glyphosate, no positive result was obtained under all experimental conditions. The number of necrotic cells was statistically significantly increased for glyphosate and Roundup at 20 mg/L. The number of apoptotic cells was statistically significantly increased with glyphosate at 20 mg/L but not with Roundup.

Discussion

The results show that Roundup but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The genotoxicity tests show that glyphosate as well as its formulation induce DNA strand breaks as well as nuclear anomalies that reflect DNA instability including chromosomal damage. Glyphosate did not induce effects in the NR, SRB, and in the XTT assay up to concentrations of 200 mg/L, while a clear effect was seen in the LHDe assay from 80 mg/L on. On the contrary, statistically significant cytotoxic effects were observed with Roundup in all four assays, and significant changes were seen in the LDHe assay from 10 mg glyphosate eq./L and in the XTT test from 40 mg glyphosate eq./L on. Comparisons of the sensitivity of the different toxicity tests indicate that Roundup causes membrane damage at lower concentrations than the inhibition of mitochondrial functions in embryonic and placental-derived cells (JEG3), while umbilical vein cord endothelial cells (HUVEC) were equally sensitive to both endpoints. High concentrations of glyphosate affected in these experiments primarily mitochondrial functions while no effects were seen in an assay detecting changes of cell membrane functions.

The results of the SCGE assays show that glyphosate as well as Roundup induce comet formation under alkaline conditions. The effects increased as a function of the exposure and DNA migration was also observed under conditions that did not affect the cellular integrity. Also in earlier investigations with human-derived cells, SCGE assays were used to monitor the DNA-damaging effects of glyphosate. Significant induction of DNA migration was seen in human hepatoma cells (HepG2) with Roundup 400 with glyphosate levels of more than 5 mg/L after 24 hours of treatment. Other cell lines such as normal human fibroblasts (GM38) and the human fibrosarcoma line HT1080 were less sensitive in long-term (72 h) exposure studies. Taken together, these effects show that distinct differences exist in the sensitivity of cells from different organs and indicate that drug-metabolizing enzymes, which are represented in the enzyme activation mix and in HepG2 cells, increase the DNA-damaging properties of glyphosate. The most relevant finding of the present study is the observation of a statistically significant, dose-dependent induction of MNi, BN-MNi, and NB reflecting the genomic damage by glyphosate and its formulation. The most sensitive endpoint was MNi induction. Treatment of the cells with highest level (20 mg glyphosate eq./L) of Roundup caused a 3-fold increase over the background. A weaker effect was seen with the corresponding concentration of glyphosate. NPB was a less responsive endpoint with a statistically significant increase with glyphosate and Roundup at all dose levels. These results indicate that the damage seen in the SCGE assays is not completely repaired but leads to persisting alterations of the genetic material. MNi reflect numerical as well as structural chromosomal aberrations, while NB are formed as a consequence of gene amplification or expulsion of intact chromosomes or fragments; NPB are caused by formation of dicentric chromosomes.

The findings of the present study suggest that buccal epithelial cells are more sensitive toward the cytotoxic and DNA-damaging effects of glyphosate and Roundup than cells from the hematopoietic system. The Roundup formulation that was tested contains 450 g/L of glyphosate and should be diluted according to the instructions of the manufacturer to 1–3 % before use (final concentration 4,500–13,500 mg/L). The fact that we found significant acute and genotoxic effects at levels between 10 and 20 mg/L after 20 min indicates that short contact with a 225–1,350-fold dilution of the formulation may cause adverse effects in cells from the oral cavity (and possibly also in other respiratory epithelia).

Assessment and conclusion

Assessment and conclusion by applicant:

The objective of the present study was to find out whether exposure of human-derived buccal epithelial TR146 cells to glyphosate and Roundup causes adverse effects. In cytotoxicity experiments, four different endpoints were used, which reflect different modes of action. To assess the effect of glyphosate and Roundup on DNA stability, single cell gel electrophoresis assays (SCGE) were conducted under standard alkaline conditions reflecting the formation of single- and double-strand breaks. Additionally cytokinesis-block MN cytome assays have been conducted in which different nuclear anomalies were measured. This study demonstrated that there is a big difference in cytotoxicity between glyphosate and Roundup. This is not surprising since the

surfactants present in glyphosate formulations decrease the integrity of cell and mitochondrial membranes causing toxicity and ensuing DNA instability. Glyphosate was found to significantly increase tail intensity as of 20 mg/L but without any further increase with dose from 40 to 2000 mg/L. Roundup increased in a dose dependent manner the tail intensity from 20 mg glyphosate eq./L up to 200 mg glyphosate eq./L with increasing cytotoxicity and 0 % cell integrity at 200 glyphosate eq. mg/L. This indicates that there is a relationship between the cytoxicity of Roundup and DNA instability. This study has demonstrated a greater sensitivity of buccal epithelial cells for glyphosate and its formulations than hematopoietic cells where no effects have been noted in *in vivo* MN tests with doses up to 2,000 mg/kg bw. Since there is no direct exposure of the buccal epithelium with the Roundup formulation (unless it is swallowed) during application and the inhalation of aerosol of the spray dilution during application is negligible (Jauhiainen A *et al*. (1991) Am. Ind. Hyg. Assoc. J. 52, 61–64) the likelihood of DNA damage in epithelial cells of the GI and the respiratory tract remains very low.

This publication is considered relevant for the risk assessment of glyphosate and reliable with restrictions. Although it complies with most of the reliability criteria of an *in vitro* toxicology study, no blinded scoring of coded slides is reported. Also, concentrations with positive findings (20-2000 mg/L) noted significant effects on necrosis and apoptosis markers in parallel experiments at the low dose of 20 mg/mL.

Reliability criteria for *in vitro* toxicology studies made by the applicant

Publication: Koller *et al.*, 2012.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 95 %. Source: Monsanto Europe S.A.
Only glyphosate acid or one of its salts is the tested substance	Y	Also formulation (Roundup Ultra Max) was tested
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	NA	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	10-1000 µg/mL
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Roundup, but not glyphosate causes pronounced cytotoxic effects in human-derived buccal epithelial cells. The alkaline comet test results show that glyphosate as well as Roundup induce comet formation that reflect strand breaks and apurinic sites.

		20 µg/mL glyphosate in Roundup caused a 3-fold increase over the background, with glyphosate alone a weaker effect was seen.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate and reliable without restrictions because it complies with most of the reliability criteria of an in vitro toxicology study. Although it complies with most of the reliability criteria of an in vitro toxicology study, no blinded scoring of coded slides is reported. Also, concentrations with positive findings (20-2000 mg/L) noted significant effects on necrosis and apoptosis markers in parallel experiments at the low dose of 20 mg/mL.		

Assessment and conclusion by RMS:

It is largely agreed with the applicant's summary of the publication as described above. However, the statement that buccal epithelium is not exposed during treatment of crops with glyphosate-containing products was not further verified by the RMS.

In contrast to the applicant, however, the RMS questions the reliability of the study due to multiple deviations from the OECD guidelines as noted above and considers the study to be supportive only.

For the sake of completeness, results of the Comet assay and the micronucleus assay were included by the RMS below since the applicant did not include any tables or figures in its summary.

According to the added figures, apoptosis seems to be pronounced already at 10 mg/L compared to the medium control and the positive control. It is therefore not unlikely that the observed positive effects observed at 10 mg/L and above are due to cytotoxicity.

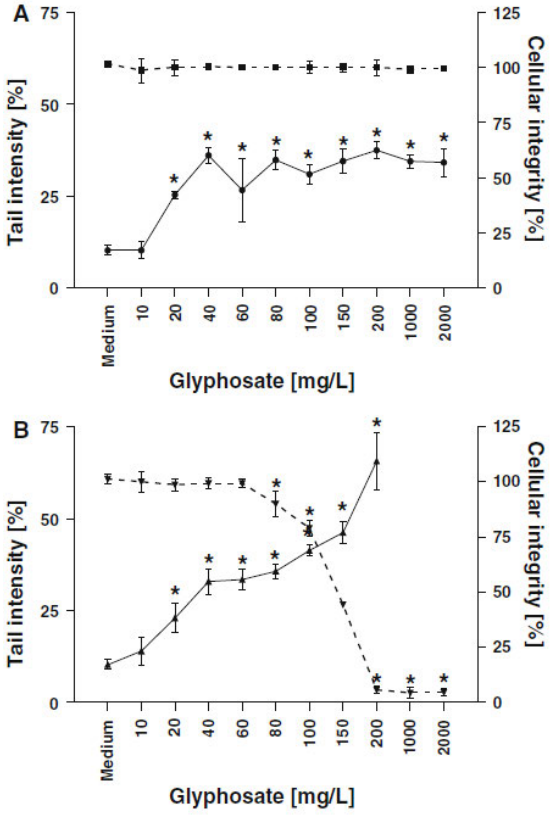


Figure B.6.4.4.13-1: Impact of G **(A)** and **R (B)** on the cellular integrity and on DNA migration in the human-derived epithelial buccal cell line TR146. The cells were treated with different concentrations of the test compounds for 20 min. Subsequently, DNA damage was analyzed, and the cellular integrity was determined. Each experimental point represents results (means ± SD) obtained with three parallel cultures. From each, 50 cells were evaluated for comet formation (solid line). The cellular integrity (dotted line) was determined with Neutral Red (data represent means of three experiments in which the means of the cellular integrity of three cultures were determined). Asterisks indicate significant differences from control values (Dunnett's test, $p \leq 0.05$)

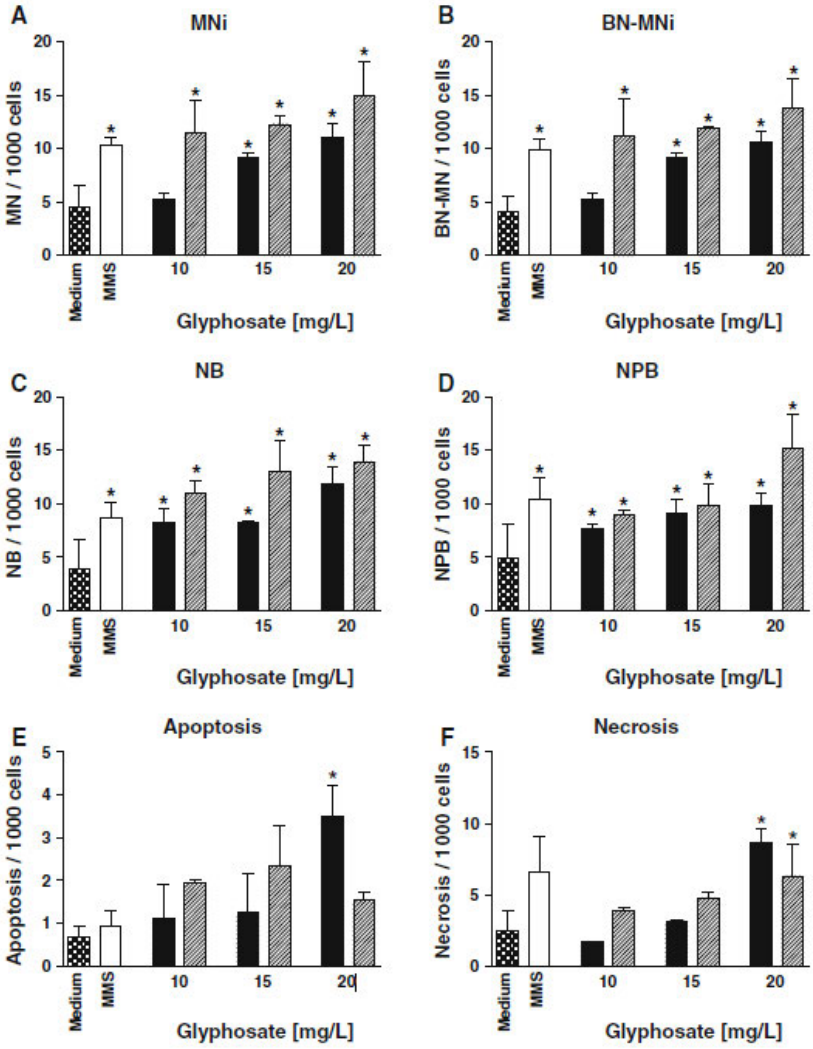


Figure B.6.4.4.13-2: Impact of G and R on the frequencies of MNi, BNMNi, NB, NPB and on apoptosis as well as necrosis in human-derived buccal epithelial cells (TR146). The cells were treated with aqueous solutions of G and R for 20 min. Subsequently, they were washed twice with DPBS, and cytochalasin B (3.0 µg/ml) was added for 48 h. The different endpoints were determined as described by Fenech (2007). Bars represent the means ± SD of results obtained with three cultures; from each, > 1,000 cells were evaluated. Black bars show findings obtained with pure G; shaded bars represent results obtained with R (the concentrations were established in such a way that the G levels in the formulation corresponded to those of the pure active component G). MMS (100 lg/ml; 20 min) was used as a positive control. Asterisks indicate statistical significance from negative control values (chi-square test with Yate's correction, $p \leq 0.001$)

B.6.4.4.14. *In vivo studies in somatic cells – 28-day oral exposure study in rats investigating oxidative stress and DNA damage – public literature study 14*

Data point:	CA 5.3.1/010
Report author	Milic, M. *et al.*

Report year	2018
Report title	Oxidative stress, cholinesterase activity, and DNA damage in the liver, whole blood, and plasma of Wistar rats following a 28–day exposure to glyphosate
Document No	DOI: 10.2478/aiht-2018-69-3114 E-ISSN: 1848-6312
Guidelines followed in study	**GRG:** None **AGG:** For the 28-day repeated oral exposure of the rats, no specific guideline was followed as this study is an investigative study. The alkaline comet assay largely follows OECD GD 489. However, several deviations were noted (based on the information provided in the publication): - No justification is provided for the harvesting timepoint at 24 hours after the last dosing. According to the OECD GD sampling time should be determined from kinetic data (e.g. at Tmax for plasma or tissue concentrations or at the steady state for multiple administrations). - Slides were left 10 minutes for unwinding of the DNA, whereas the OECD GD states for at least 20 minutes. - The frequency of hedgehogs was determined based on visual scoring of 100 nucleoids per sample instead of 150. Further, the data on frequency of hedgehogs was not reported in the publication. - No data on the proficiency of the lab for performing the alkaline comet assay has been provided in the publication (e.g. no historical control data on the positive and negative is provided).
Deviations from current test guideline	No
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability:	**Conclusion GRG:** Yes/Reliable with restrictions **Conclusion AGG**: The study is considered as supportive only as the alkaline comet assay was not fully guideline compliant (refer to deviations above) and the other parts of the study are considered reliable with restrictions.

Full summary of the study according to OECD format

In this 28 day-study, the effects of the herbicide glyphosate administered by gavage to Wistar rats at daily doses equivalent to 0.1 of the acceptable operator exposure level (AOEL), 0.5 of the consumer acceptable daily intake (ADI), 1.75 (corresponding to the chronic population-adjusted dose, cPAD), and 10 mg kg-1 body weight (bw) (corresponding to 100 times the AOEL) were evaluated. At the end of each treatment, the body and liver weights were measured and compared with their baseline values. DNA damage in leukocytes and liver tissue was estimated with the alkaline comet assay. Oxidative stress was evaluated using a battery of endpoints to establish lipid peroxidation via thiobarbituric reactive substances (TBARS) level, level of reactive oxygen species (ROS), glutathione (GSH) level, and the activity of glutathione peroxidase (GSH-Px). Total cholinesterase activity and the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were also measured. The exposed animals gained less weight than control. Treatment resulted in significantly higher primary DNA damage in the liver cells and leukocytes. Glyphosate exposure significantly lowered TBARS in the liver of the AOEL, ADI, and cPAD groups, and in plasma in the AOEL and cPAD group. AChE was inhibited with all treatments, but the AOEL and ADI groups significantly differed from control. Total ChE and plasma/liver ROS/GSH levels did not significantly differ from control, except for the 35 % decrease in ChE in the AOEL and ADI groups and a significant drop in liver GSH in the cPAD and 100xAOEL groups. AOEL and ADI blood GSH-Px activity dropped significantly, but in the liver it significantly

increased in the ADI, cPAD, and 100xAOEL groups vs. control.

Materials and methods

Chemicals – Glyphosate of analytical standard purity grade (purity of ≤100 %) was purchased under the brand name PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany.

Animals - Thirty male Wistar rats were obtained from the breeding unit at Institute for Medical Research and Occupational Health (IMROH), Zagreb, Croatia and were kept under pathogen-free, steady-state microenvironmental conditions in polycarbonate cages with 40-60 % humidity at 22 °C and normal 12-hour light/dark cycle. The animals had free access to standard Good Laboratory Practice-certified food and tap water. At 3 months of age, the rats were weighted, inspected, and judged to be healthy and fit for the experiment by a licensed veterinarian at IMROH.

Experimental design - Rats were randomly divided into 6 dose groups of 5 animals each and treated orally by gavage for 28 days. Glyphosate was tested by gavage at 0.1, 0.5, 1.75 and 10 mg/kg bw/day, doses corresponding respectively with the AOEL from EFSA, the ADI, the chronic population-adjusted dose (cPAD) from EPA and 100 times the AOEL. Ethyl methanesulfonate (EMS) dissolved in PBS served as the positive control and was administered at 300 mg/kg bw over the last 3 days of the experiment. PBS served as the negative control. Body weight was monitored once a week and the glyphosate doses adjusted accordingly. Survival and clinical signs of toxicity were monitored on a daily basis. All animals were humanely sacrificed and dissected on day 29, 24 hours after the last dose. All animals were subjected to necropsy and examined for gross pathological changes.

Sample collection and preparation - Liver was excised, rinsed with cold PBS (pH 7.4) and weighed. Livers were then washed in cold TBS buffer (pH 7.5) to remove as much blood as possible, homogenised in a 50 mM potassium PBS (pH 7.4) with 1 mM EDTA, and centrifuged at 20,000 x g at 4°C for 30 minutes to obtain the supernatant. In a separate procedure, a small portion of the liver tissue was minced in a chilled mincing solution to obtain a cellular suspension. The cell suspension was left a few seconds for large clumps to settle, and the supernatant was used to prepare comet slides. This was performed within 60 minutes from sacrifice to avoid confounding necrotic changes. Blood samples were collected directly from the heart into heparinised vacutainers with an extra addition of Li-heparin and mixed vigorously. Samples were then kept at 4°C pending further processing. Plasma for the biochemical assays was prepared by centrifugation of heparinized blood at 976 x g at 4°C for 10 minutes and kept at -20°C pending analysis.

Alkaline comet assay in leucocytes and liver cells - Two microgels were prepared per tissue per animal. Slides were marked with a randomly generated code. For each slide, an aliquot of 10 µL of the cell suspension was mixed with low melting point agarose (LMPA, 0.5 %) dissolved in TBS buffer. "Sandwich" agarose microgels made of four layers were prepared on microscopic slides. Slides were pre-coated with 1 % normal melting point agarose (NMPA) and air-dried. The second gel layer of 0.6 % NMPA was then applied. The third layer consisted of 0.5 % LMPA mixed with heparinized whole blood (10 µL per slide) or 10 µL of liver cell suspension per slide. Finally, 0.5 % LMPA was applied as the top layer over the gel-embedded cells. After solidification of the gel on ice-cold metal tray, the slides were submerged in freshly prepared cold lysing solution (pH 10) at 4°C overnight. The slides were quickly washed with distilled water and left in a vertical Coplin jar with chilled electrophoresis buffer (pH >13) at 4°C for 10 minutes. The slides were then transferred into a horizontal electrophoresis unit. After electrophoresis, the slides were washed 3 times with neutralisation buffer (pH 7.5). All gels were dehydrated with 70 % and 96 % ethanol, respectively, air dried, and stored at room temperature. Before scoring, the slides were stained with ethidium bromide (20 µg/mL) and analysed with a fluorescent microscope at 200x magnification using the Comet Assay IV image analysis system equipped with appropriate filters. Three hundred cells (2 x 150 nucleoids) were scored in total for each animal and sample. Medium-sized cells (parenchymal cells or hepatocytes, between 30 and 40 µm of head length) and small-sized cells (non-parenchymal cells, <30 µm of head length) were recorded separately. Areas

near slide margins were not scored. DNA damage was measured as comet DNA tail intensity (% of DNA in tail) and tail length (TL, expressed in µm, measured from the estimated edge of the comet head). Comets with small or non-existing head and large, diffuse tails (cells with >80 % DNA in the tail) were excluded from analysis. The frequency of such comets ("hedgehogs" or "clouds") was determined based on visual scoring among 100 nucleoids per sample.

Determination of ROS levels in plasma and liver - ROS levels in plasma and liver homogenates were measured using 2′,7′-dichlorofluorescein diacetate (DCF-DA). The acetate group of DCFH-DA gets DCFH-DA into the cells or organelles, and once inside, it is removed by cellular esterases, producing reduced DCFH which then can be oxidised by peroxides to form fluorescent oxidised DCF that can be measured spectrophotometrically. Plasma samples and 1 % liver tissue homogenate were prepared by dilution with ice-cold PBS (pH 7.4). Black 96-well plates were filled with 0.07 mL of PBS, and 0.03 mL of 1 % liver tissue homogenate or with 0.1 mL of 10 % plasma in quintuplicate for each glyphosate concentration and sample type. 20 µL of 0.12 mM DCFH-DA dye in PBS was then added to each well, and the plates were incubated at 37°C for 30 minutes. Control for dye autofluorescence was prepared without the addition of dye. Control samples were included in each experiment. Samples were analysed using a Victor3™ multilabel plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. The readings were expressed as relative fluorescence units (RFU).

Determination of lipid peroxidation in plasma and liver - The concentration of thiobarbituric reactive substances (TBARS), as a measure of lipid peroxidation, was determined. 5 µL of butylated hydroxytoluene (BHT, 0.2 % w/v) and 750 µL of phosphoric acid (1 % v/v) were added to 50 µL of sample. After mixing, 250 µL of thiobarbituric acid (TBA, 0.6 % w/w) and 445 µL of water were added, and the reaction mixture was incubated at 90°C for 30 minutes. The mixture was cooled, and absorbance measured at 532 nm using a Shimadzu UV probe spectrophotometer. TBARS concentrations were calculated using the standard curves for 1,1,3,3-tetramethoxypropane, obtained by increasing its concentrations, and expressed as µM.

Determination of GSH in plasma and liver – Glutathione (GSH) levels were analysed with a fluorogenic bimane probe using monochlorobimane (MBCl), which reacts specifically with GSH to form a fluorescent adduct. Plasma samples and liver tissue homogenates were prepared as previously described for ROS determination. 20 µL of 0.24 mM MBCl dye in PBS was then added to react at 37 °C for 20 minutes. The amount of GSH in the samples was analysed using a Victor3™ multilabel plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Control samples were included in each experiment. The readings were expressed as relative fluorescence units (RFU). Each sample analysis was performed in quintuplicate.

Determination of GSH-Px activity in whole blood and liver – Glutathione peroxidase (GSH-Px) activity in whole blood and in the supernatant of liver homogenate were determined in accordance with the European standardised method. The amount of GSH oxidised by t-butyl hydroperoxide was determined based on the decrease in β-NADPH absorbance at 340 nm, measured by spectrophotometry. One unit of GSH-Px was the number of micromoles of β-NADPH oxidised per minute. Its activity in whole blood was expressed per gram of haemoglobin (U g_{Hb}^{-1}), and in liver homogenate per gram of total protein (U $g_{protein}^{-1}$).

Protein quantification - Proteins were quantified according to the method of Bradford, using bovine serum albumin as the standard.

Cholinesterase activity in plasma - Plasma samples were analysed for total ChE, AChE, and BChE activities in a 0.1 M sodium phosphate buffer (pH 7.4) at 25°C using ATCh (1.0 mM) and DTNB (0.3 mM) as described by Ellman *et al*. To distinguish between AChE and BChE activities the BChE-selective inhibitor ethopropazine (20 µM) was used. Increase in absorbance was monitored at 412 nm over 4 minutes by means of a Cecil 9000 spectrophotometer. Enzyme activities were expressed as IU

g/protein.

Statistical analysis - Statistical analysis was run on Dell Statistica software STATISTICA, version 13.2. The data were first evaluated with descriptive statistics. The results were expressed as means ± standard deviation, and for the comet assay also medians and ranges (min-max) were used. Relative liver weights were logarithmically transformed and analysed with one-way ANOVA. For pairwise organ comparison the *post-hoc* Tukey's HSD test was used. Normality was tested with the Levene's test. Since the results of the alkaline comet assay were not normally distributed even after logarithmic transformation, the non-parametric Mann-Whitney U test was used. For multiple comparisons of cholinesterase activities, TBARS, and GSH-Px activities between the glyphosate and control groups the Kruskal-Wallis test was used. ROS and GSH levels were compared between the groups using the non-parametric Mann-Whitney U test. P values of ≤ 0.05 were considered statistically significant.

Results and Discussion

Changes in body and liver weight - No premature death or any clinical signs of systemic toxicity in male adult Wistar rats was observed in any of the groups dosed with glyphosate for 28 days. Gross necropsy did not reveal any treatment-related findings. All glyphosate-treated animals showed a similar weight gain throughout the 28–day treatment period, with significant difference between the day before treatment and at the end of treatment. Compared to the negative control animals, the glyphosate-treated animals gained considerably less body weight. There were no statistically significant changes in relative liver weight.

Alkaline comet assay in leucocytes and liver cells - Glyphosate-treated rats had higher primary DNA damage in leukocytes when compared to control in tail length and tail intensity. While tail length was significantly greater at all dose levels, only the lowest dose tested resulted in significantly higher mean tail intensity. One reason for that could be high standard deviations. It is worth noting that glyphosate caused greater DNA damage in liver cells than in the leukocytes. The greatest liver cell DNA damage (as tail intensity) occurred at the lowest and the highest dose both in parenchymal and non-parenchymal liver cells. In fact, the group exposed at 10 mg/kg bw/day suffered even greater damage in medium-sized liver cells than the positive control.

Table 6.3.1.8-1. Results of the alkaline comet assay in adult male Wistar rats (N=5 per group) orally treated with different doses of glyphosate for 28 days (table added by the RMS)

Cell type		Mean ± SD Median Range	Negative control	Positive control (EMS, 300 mg/kg bw/day)	0.1 mg/kg bw/day	0.5 mg/kg bw/day	1.75 mg/kg bw/day	10 mg/kg bw/day
Leukocytes		**TL**	16.92±3.30[PC] 15.83 11.25–35.42	29.84±10.87[NC] 27.92 10.42–75.42	19.49±3.83[PC, NC] 18.75 11.67–40	19.06±3.50[NC, PC] 17.92 11.25–32.08	19.46±3.47[NC, PC] 18.75 11.25–36.67	18.44±4.02[NC, PC] 16.67 11.25–36.25
		TI	0.96±1.75[PC] 0.07 0–11.34	7.93±7.69[NC] 5.58 0–39.33	1.04±1.95[NC, PC] 0.06 0–10.19	1.23±2.28[PC] 0.06 0–12.64	1.17±2.06[PC] 0.09 0–10.87	1.02±1.82[PC] 0.10 0–10.24
Liver cells	**Small sized nuclei**	**TL**	18.34±4.05[PC] 17.08 5.83–38.33	23.23±3.92[NC] 22.92 12.5–36.25	23.65±8.01[NC, PC] 20.83 11.67–52.5	25.36±7.32[NC, PC] 23.75 10–54.17	25.27±8.54[NC, PC] 23.33 11.67–65.42	28.17±9.40[NC, PC] 27.08 7.92–54.58
		TI	0.87±1.69[PC] 0.07 0–14.74	4.10±3.26[NC] 3.26 0–16.19	4.37±6.26[NC, PC] 1.03 0–32.28	2.89±3.70[NC, PC] 1.31 0–21.39	2.47±3.39[NC, PC] 0.77 0–19.03	4.25±4.48[NC, PC] 2.64 0–24.32
	Medium sized nuclei	**TL**	21.26±4.07[PC] 20.42 11.67–48.33	23.13±4.07[NC] 25.42 12.5–39.17	26.69±11.24[NC, PC] 21.67 11.25–61.25	29.27±8.59[NC, PC] 27.92 10.83–54.58	28.74±8.30[NC, PC] 27.5 15–60.83	32.59±10.16[NC, PC] 32.08 12.92–63.75
		TI	0.90±1.71[PC] 0.05 0–9.81	3.10±2.99[NC] 1.96 0–16.32	4.97±7.13[NC, PC] 0.65 0–37.45	3.06±3.82[NC, PC] 1.38 0–21.51	2.17±2.89[NC, PC] 0.77 0–15.89	4.54±4.65[NC, PC] 3.16 0–21.89

A non-parametric Mann Whitney U test was used for comparison between different treatment groups. Results are presented as mean ±S.D., median and range (min-max). TL – tail length in µm; TI – tail intensity (%DNA in tail); NC – significantly different from negative control; PC – significantly different from positive control; Statistical significance was set at $p<0.05$.

ROS levels in plasma and liver - No statistically significant difference was found in ROS levels in plasma between the glyphosate dose groups and control. A small decrease in ROS of around 7 % was seen in the groups with the highest glyphosate doses. Liver tissue showed a similar pattern with a decrease in ROS at the highest dose levels which was statistically significant at 10 mg/kg bw/day. ROS levels in the liver were 100 times greater than in the plasma.

Lipid peroxidation in plasma and liver - TBARS concentrations in plasma and liver were decreased in all glyphosate-dosed groups when compared to control. However, without a dose-response relationship.

GSH levels in plasma and liver – In plasma, no statistically significant difference was evident between the glyphosate-dosed groups and the control group. In the liver, however, a statistically significant decrease in GSH was seen in the two highest dose groups.

GSH-Px activity in whole blood and liver - GSH-Px activity in whole blood was statistically significantly decreased at 0.5 and 1.75 mg/kg bw/day but not at 10 mg/kg bw/day. In contrast, GSH-Px activity in the liver was statistically significantly increased at 0.5, 1.75 and 10 mg/kg bw/day.

Cholinesterase activity in plasma - Glyphosate did not significantly affect total cholinesterase (ChE) activity, even though there was a decrease about 35 % at 0.1 and 0.5 mg/kg bw/day when compared to control. Acetyl cholinesterase (AChE) activity in turn, was statistically significantly decreased at 0.1 and 0.5 mg/kg bw but not at the higher dose levels. There was no statistically significant change in activity of butyl cholinesterase in the glyphosate dose groups when compared to the control group.

Discussion and conclusion

This study suggests that sub-chronic exposure to glyphosate mostly affects DNA in the liver and white blood cells. General oxidative stress was not confirmed, while total cholinesterase activity showed some, but inconsistent, changes from control. Exposure to environmentally relevant levels of glyphosate, presumably not harmful to humans, seems to have different effects from exposure to much higher doses, especially where oxidative stress is concerned. In this study it has been demonstrated that even without evidence of oxidative stress, small doses allowed for human exposure can produce significant primary DNA damage and inhibit AChE, which may both be related to indirect action through glycine substitution.

Assessment and conclusion by applicant:

Glyphosate was orally administered to male rats at 0.1, 0.5, 1.75 and 10 mg/kg bw for 28 days to investigate its effect on DNA damage, oxidative stress and cholinesterase activity. The endpoints of this study were DNA damage as measured in the alkaline comet assay, ROS in plasma and liver, lipid peroxidation in plasma and liver, GSH in plasma and liver, GSH-Px activity in whole blood and liver and total cholinesterase, acetyl cholinesterase and butyl cholinesterase activity in plasma. The results of the alkaline comet assays revealed a statistically significant increase in tail length and tail intensity in leucocytes and small and medium sized liver nuclei. With the exception of tail length of small sized liver nuclei no dose-effect relationship was evident. Tail intensity of the leucocytes could not be assessed because of the very high variability of the results. From the results of the oxidative stress markers in plasma and liver and cholinesterase activity in plasma it can be concluded that there was no dose-related effect. In summary, the results do not allow a conclusion on effects of glyphosate on DNA damage, oxidative stress and cholinesterase activity.

Reliability criteria for *in vivo* toxicology studies made by the applicant

Publication: Milic *et al.*, 2018.	**Criteria met? Y/N/?**	**Comments**
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	Y	*In vivo* Comet assay was compliant with OECD

		TG 489.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of ≤100 %). Source: PESTANAL®, a registered trademark of Sigma-Aldrich Laborchemikalien GmbH, Germany
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	0.1, 0.5, 1.75 and 10 mg/kg bw/day, positive and negative control included.
Number of animals used per dose level reported	Y	5 males per dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	Limited to body weight, organ weight, DNA damage, oxidative stress and ChE activity.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the high variability of the results of the comet assay, although the conduct of this assay was in general compliant with OECD test guideline 489.		

Assessment and conclusion by RMS:

The alkaline comet assay was not fully guideline compliant and due to the low top dose and the deviations considered as not acceptable. Based on the information provided it is not possible to determine whether the acceptance criteria of the assay are met as no information is provided on the compatibility of the positive and negative controls with the laboratory's historical control database. Therefore it is not possible to determine whether the outcome of the assay should be considered positive, negative or equivocal. The only conclusion that can be drawn is that with the exception of tail length of small sized liver nuclei, no dose-effect relationship was evident. In addition, it is noted that – besides the reported deviations from the test guideline - the high variability in the results makes the interpretation of the results hardly possible.

Considering the other investigated parameters, the RMS agrees with the assessment of the applicant that from the results of the oxidative stress markers in plasma and liver and cholinesterase activity in plasma it can be concluded that there was no dose-related effect. In addition, body weight gain was lower in the treated animals, however, without a dose-response relationship. No changes were observed in relative liver weight.

B.6.4.4.15. *Public literature referenced in RAC opinion (RAC 40, 2017) in human populations*

In Volume 1, section 2.6.4.1 reference is also made to the RAC opinion (RAC 40, 2017) in which some genotoxicity studies in human population after occupational exposure to glyphosate-based herbicides (GBH) or exposure of bystanders/area residents exist. RAC mentioned that, however, some evidence was suggested in two published studies which investigated populations believed to be exposed to glyphosate based formulations.
The applicant did not submit these studies, as the studies were published over 10 years ago which is outside the scope of the current public literature search. For completeness, RMS has included the summaries and evaluations of these two studies from the previous RAR (2015) and these are shown below. RMS has **not** re-evaluated this information as these studies were considered not reliable and not relevant during the previous assessment.

1) Paz-y-Miño and co-workers (2007), as described in the previous RAR (2015):

Author(s)	Year	Study title
Paz-Y-Mino, C. Sanchez, M. E. Arevalo, M. Munoz, M. J. Witte, T. De-La-Carrera, G. O. Leone, P. E.	2007	Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genetics and Molecular Biology Volume: 30 Number: 2 Pages: 456-460 ASB2012-11992

Abstract*
We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the comet assay. The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5 mu m) compared to the control group (comet length = 25.94 mu m). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Documentation of Comet assay insufficient for assessment.
Relevance of study:	Not relevant (Glyphosate formulation was applied at much higher dose rates than recommended for the intended uses in the EU. In addition, the herbicide was combined with the adjuvant (Cosmoflux 411F) that can increase the biological action of the herbicide. This adjuvant will not be used in the EU.)
Klimisch code:	3

Exposed Population	End point	Exposures	Result	Reference
Individuals on or near glyphosate spraying	Lymphocyte alkaline SCGE	Glyphosate formulation aerially sprayed within 3 km	Statistically significant increases in damaged cells	Paz-y-Mino C, 2007 (ASB2012-11992)

"One publication reported induction of alkaline SCGE effects in blood lymphocytes of populations living within 3 km of areas sprayed with glyphosate formulation for illicit crop eradication (Paz-y-Mino C, 2007, ASB2012-11992). The populations studied were relatively small (24 exposed individuals and 21 non-exposed individuals). The sprayed material was reported to be Roundup Ultra, a GBF containing 43.9 % glyphosate, polyethoxylated tallowamine surfactant and a proprietary component, Cosmoflux 411F. Specific methods for collection, storage, and transport of blood samples are not described for either the exposed population or control group. The publication also does not indicate that slides were coded for scoring which consisted of visual classification into

damage categories and measurement of DNA migration (tail length). There were fairly large differences in ages and sex distribution of the exposed and control populations but these did not appear to be statistically significant. The study reported increases in damaged cell categories and statistically significant increases in DNA migration (tail length) in the presumably exposed population. Interpretation of the results of this study should consider numerous reported signs of toxicity in the exposed population and the reported application rate of 24.3 litres/ha which was stated to be 20 times the maximum recommended application rate. Some of the reported human health effects described by Paz-y-Mino (2007, ASB2012-11992) appear to be consistent with severe exposures noted in clinical reports of acute poisoning incidents with GBFs and other pesticide formulations (often self-administered) rather than typical bystander exposures. Given the considerably favorable general toxicology profile of glyphosate as reported by the WHO/FAO Joint Meeting on Pesticide Residues (WHO/FAO, 2004, ASB2008-6266) and in Williams et al. (2000, ASB2012-12053), factors related to either high surfactant exposure, unusual GBF components in this formulation or other undocumented variables appear to be confounding factors in this study. It appears that the reported alkaline SCGE effects could well have been secondary to the ailments reported in this study population. "

2) Bolognesi and co-workers (2009), as described in the previous RAR (2015):

Exposed Population	**End point**	**Exposures**	**Result**	**Reference**
Humans in areas where glyphosate formulation is applied	Lymphocyte cytokinesis block micronucleus (CB MN)	Aerial or manual spraying of glyphosate formulation for illicit crop control and sugar cane maturation	Increase in CB MN but no clear relationship to assumed or reported exposures	Bolognesi et al., 2009 (ASB2012-11570)
Agricultural workers	Buccal cell micronucleus	Glyphosate formulation use reported along with numerous other pesticides	Statistically significant increase in MN	Bolognesi et al., 2009 (ASB2012-11570)

"A second publication reported results for a blood lymphocyte cytokinesis-block micronucleus study of individuals in areas treated with glyphosate formulation by aerial spraying or manual application (Bolognesi et al., 2009, ASB2012-11570). Although the title of the publication contains the term "agricultural workers", most of the populations studied do not appear to be agricultural workers who are involved in application of GBFs. The human lymphocyte culture and scoring methodology employed in the Bolognesi et al. (2009, ASB2012-11570) study appear to be generally consistent with commonly used and recommended practices for this assay. However, there is a significant question as to how long the blood samples used in the study were stored prior to initiating cultures and this may have affected the micronucleus numbers observed in the different sets of samples and populations. Also, the populations in the aerially sprayed regions had a second sampling a few days after the first sampling and this second sampling was not performed in the control populations. The publication reported a small increase in the frequency of binucleated cells with micronuclei and micronuclei per cell in samples collected from people living in three regions after spraying of GBFs compared with control values of samples collected just before spraying. However, the pattern of the increases did not correlate either with the application rate or with self-reported exposure. The largest post-spraying increase in binuclated cell micronucleus frequency was reported for a population with a much lower glyphosate active ingredient application rate and only 1 of 25 people in this region reported contact with sprayed glyphosate formulation. Increases in binucleated cell micronucleus frequency did not have a statistically significant relationship with self-reported exposure for two other populations. Some interpretative statements in Bolognesi et al. (2009, ASB2012-11570) suggest a small transient genotoxic effect of glyphosate formulation spraying on frequencies of binucleated cells with micronuclei, but other statements indicate that causality of the observed effects could not be determined using reasonable criteria and that lack of exposure data precluded conclusions. This study has a combination of uncontrolled or inadequately characterized variables, such as uncharacterised exposure to "genotoxic pesticides", that would appear to preclude using the data to support any conclusion that exposure to GBFs affects binucleated micronucleus frequencies. Actually, the available data, while certainly limited in nature, support a conclusion that the observed effects do not appear to be attributable to glyphosate formulation exposure."

B.6.4.4.16. *Public literature referenced in previous CLH report (BAuA, May 2016) on in vitro and in vivo genotoxicity studies*

In Volume 1, section 2.6.4.1 reference is also made to the previous CLH report (BAuA, May 2016) in which some additional *in vitro* and *in vivo* genotoxicity studies were discussed.
The applicant did not submit these studies, as the studies were published over 10 years ago which is outside the scope of the current public literature search and as the studies were considered not reliable/relevant or of limited reliability/relevance during the previous assessment. For completeness, RMS has included the summaries and evaluations of these ~~two~~ studies from the previous RAR (2015) and these are shown below. RMS has **not** re-evaluated this information as these studies were considered not reliable/relevant or of limited reliability/relevance during the previous assessment.

In vitro

Lioi et al. 1998 (ASB2013-9836 and ASB2013-9837), as described in the previous RAR (2015):

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
Chromosome aberration (CA) and Sister chromatid exchange (SCE) in human lymphocytes	Glyphosate (purity ≥ 98 %)	0-5.0-8.5-17.0-51.0 µM	Increase in CA and SCE frequency	Increase of SCE not dose related in highest dose group	Lioi et al, 1998a ASB2013-9836
CA and SCE in bovine lymphocytes	Glyphosate (purity ≥ 98 %)	0-17-85-170 µM	Increase in CA and SCE frequency	Increase of SCE not dose related in highest dose group	Lioi et al, 1998b ASB2013-9837

Lioi et al. (1998a, ASB2013-9836 and 1998b, ASB2013-9837) reported an increase in CA and SCE frequency in human lymphocytes of 3 donors in concentrations between 5 and 51 µM and in bovine lymphocytes between 17 and 170 µM.
Comment: The results are questionable because a number of well performed and validated studies in vitro in mammalian cells and in vivo in mammals did not register comparable effects even in dose levels more than 10 times higher than the doses used in the studies described by Lioi et al. (1998a and 1998b, ASB2013-9836 and 1998b, ASB2013-9837). A replication would be needed to confirm such aberrant results.

Mladinic et al. 2009 (ASB2012-11907) and Mladinic et al. 2009 (ASB2012-11906), as described in the previous RAR (2015):

Author(s)	Year	Study title
Mladinic, M., Perkovic, P., Zeljezic, D.	2009b	Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay Toxicology Letters Volume: 189 Number: 2 Pages: 130-137 ASB2012-11907

Abstract*
Possible clastogenic and aneugenic effects of pesticides on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure were evaluated with and without the use of metabolic activation (S9). To get a better insight into the content of micronuclei (MN) and other chromatin instabilities, lymphocyte preparations were hybridized using pancentromeric DNA probes. Frequency of the MN, nuclear buds (NB) and nucleoplasmic bridges (NPB) in cultures treated with glyphosate slightly increased from 3.5 µg/mL onward. Presence of S9 significantly elevated cytome assay parameters only at 580 µg/mL. No concentration-related increase of centromere (C+) and DAPI signals (DAPI+) was observed for glyphosate treatment. Terbuthylazine treatment showed a dose dependent increase in the number of MN without S9 significant at 0.0008 µg/mL and higher. At concentration lower than 1/16 LD_{50} occurrence of C + MN was significantly elevated regardless of S9, but not dose related, and in the presence of S9 only NBs containing centromere signals were

observed. Carbofuran treatment showed concentration dependent increase in the number of MN. The frequency of C + MN was significant from 0.008 µg/mL onward regardless of S9. Results suggest that lower concentrations of glyphosate have no hazardous effects on DNA, while terbuthylazine and carbofuran revealed a predominant aneugenic potential.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Non-GLP, non-guideline study *in vitro*. Positive and negative control results almost indistinguishable for MN assay without metabolic activation. Negative control NB and NBP results not reported.
Relevance of study:	Not relevant (Proposed mechanism of genotoxicity (*in vitro*) is not relevant to human exposure levels. Authors express confidence that estimated maximum human exposure levels correspond to acceptable safety levels based on evaluated *in vitro* endpoints, and that their findings need to be verified *in vivo*.)
Klimisch code:	3

Author(s)	Year	Study title
Mladinic, M. Berend, S. Vrdoljak, A.L. Kopjar, N. Radic, B. Zeljezic, D.	2009	Evaluation of Genome Damage and Its Relation to Oxidative Stress Induced by Glyphosate in Human Lymphocytes *in Vitro* Environmental and Molecular Mutagenesis Volume: 50 Number: 9 Pages: 800-807 ASB2012-11906

Abstract*
In the present study we evaluated the genotoxic and oxidative potential of glyphosate on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure. Testing was done with and without metabolic activation (S9). Ferric-reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and the hOGG1 modified comet assay were used to measure glyphosate's oxidative potential and its impact on DNA. Genotoxicity was evaluated by alkaline comet and analysis of micronuclei and other nuclear instabilities applying centromere probes. The alkaline comet assay showed significantly increased tail length (20.39 µm) and intensity (2.19 %) for 580 µg/mL, and increased tail intensity (1.88 %) at 92.8 µg/mL, compared to control values of 18.15 µm for tail length and 1.14 % for tail intensity. With S9, tail length was significantly increased for all concentrations tested: 3.5, 92.8, and 580 µg/mL. Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91 µg/mL with S9 and 580 µg/mL without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5 µg/mL and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580 µg/mL. FRAP values slightly increased only at 580 µg/mL regardless of metabolic activation, while TBARS values increased significantly. Since for any of the assays applied, no clear dose-dependent effect was observed, it indicates that glyphosate in concentrations relevant to human exposure do not pose significant health risk.
* Quoted from article

Klimisch evaluation

Reliability of study:	Reliable with restrictions
Comment:	Non-GLP, non-guideline *in vitro* study, meeting scientific principles
Relevance of study:	Relevant with restrictions (Assessment of Genotoxicity *in vitro* at concentrations relevant to human exposure levels; authors state

that no clear dose-dependent effect was observed, and results indicate that glyphosate in concentrations relevant to human exposure do not pose significant health risk.

Klimisch code: 2

End point	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009 (ASB2012-11907)
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009 (ASB2012-11906)

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.

[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.

[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

Mañas et al. 2009 (ASB2012-11892), as described in the previous RAR (2015):

Author(s)	Year	Study title
Manas, F. Peralta, L. Raviolo, J. Garcia Ovando, H. Weyers, A. Ugnia, L. Gonzalez Cid, M. Larripa, I. Gorla, N.	2009	Genotoxicity of glyphosate assessed by the comet assay and cytogenic tests Environmental Toxicology and Pharmacology Volume: 28 Pages: 37-41 ASB2012-11892

Abstract*
It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42±1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p < 0.01$) with glyphosate at a range concentration from 3.00 to 7.50mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20–6.00mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT) was evaluated in mice at three doses rendering statistical significant increases at 400 mg/kg (13.0±3.08 micronucleated erythrocytes/1000 cells, $p < 0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.
* Quoted from article

Klimisch evaluation

Reliability of study: Not reliable

Comment: Guideline deviations and reporting deficiencies. Several parameters in the MNT not reported. Blind scoring reported

for the CA but not MNT. Exposure route used in the MNT is not relevant for human exposure. (see guideline deviations). No indication of pH or osmolality control for the comet assay. Results not reported separately for replicates.

Relevance of study: Not relevant (Due to guideline deviations and reporting deficiencies).

Klimisch code: 3

End point	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
Chromosome aberration	Human lymphocytes	Glyphosate (96%)	6 mM (not toxic)	Negative	MA, IC, RE	Manas et al., (2009 ASB2012-11892)
Alkaline single cell gel electrophoresis (SCGE, comet)	Hep-2 cells	Glyphosate (analytical, 96%)	7.5 mM (limited by toxicity)	Positive	MA, PH, RE	Manas et al., 2009 (ASB2012-11892)

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.

[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.

[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

Bolognesi et al. 1997 (Z59299), as described in the previous RAR (2015):

Author(s)	**Year**	**Study title**
Bolognesi, C. Bonatti, S. Degan, P. Gallerani, E. Peluso, M. Rabboni, R. Roggieri, P. Abbondandolo, A.	1997	Genotoxic activity of glyphosate and its technical formulation roundup Journal of Agricultural and Food Chemistry Volume: 45 Pages: 1957-1962 Z59299

Abstract*
Glyphosate (N-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated in vivo in bone marrow of mice as micronuclei frequency and in vitro in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances *in vivo* and *in vitro*. A weak increment of the genotoxic activity was evident using the technical formulation.
* Quoted from article

Klimisch evaluation

Reliability of study: Not reliable

Comment:	Basic data given, however, the study is performed with methodological and reporting deficiencies (only data without metabolic activation generated in *in vitro* tests, no positive controls included in *in vitro* SCE and *in vivo* experiments, in some experiments only two test substance concentrations tested).
Relevance of study:	Not relevant (Due to methodological and reporting deficiencies data considered to be supplemental information; i.p. exposure route is not relevant for human exposure)
Klimisch code:	3

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
SCE assay in human lymphocytes	Glyphosate a.i. (99.9% pure)and Roundup (IPA salt with 30.4% glyphosate a.e.; alkyle sulfate surfactant)	0 - 6 mg/ml for glyphosate; 0, 0.1, 0.33 mg/ml for Roundup	Positive for glyphosate from 1 mg/ml onwards and for Roundup at both concentrations. With Roundup, complete cytotoxicity at concentrations >0.33 mg/ml.	Insufficient data. In addition, a positive result in this assay is of equivocal biological significance against the background of more appropriate mutagenicity studies.	Bolognesi et al., 1997 Z59299

Monroy et al. 2005 (ASB2012-11910), as described in the previous RAR (2015):

End point	**Test System**	**Test Material**	**Maximum Dose**	**Result**	**Comment[a]**	**Reference**
Alkaline SCGE	GM38 human fibroblasts and HT1090 human fibrosarcoma	Glyphosate (technical grade)	6.5 mM	Positive	MA, PH, TO, SC, RE	Monroy et al., 2005 (ASB2012-11910)

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.

[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.

[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

"Positive SCGE results were observed for two mammalian cell lines exposed to glyphosate for 4 hours at concentrations of 4.5-6.5 mM (GM39 cells) and 4.75-6.5 mM (HT1080 cells) (Monroy et al., 2005, ASB2012-11910). These concentrations are close to the upper limit dose of 10 mM generally recommended for *in vitro* mammalian cell assays and control of medium pH is not indicated. Characterisation of nuclear damage was done by visual scoring without coding of slides being indicated."

***In vivo* micronucleus**

Mañas et al. 2009 (ASB2012-11892), as described in the previous RAR (2015):

Author(s)	Year	Study title
Manas, F. Peralta, L. Raviolo, J. Garcia Ovando, H. Weyers, A. Ugnia, L. Gonzalez Cid, M. Larripa, I. Gorla, N.	2009	Genotoxicity of glyphosate assessed by the comet assay and cytogenic tests Environmental Toxicology and Pharmacology Volume: 28 Pages: 37-41 ASB2012-11892

Abstract*
It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42±1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p < 0.01$) with glyphosate at a range concentration from 3.00 to 7.50mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20–6.00mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT) was evaluated in mice at three doses rendering statistical significant increases at 400 mg/kg (13.0±3.08 micronucleated erythrocytes/1000 cells, $p < 0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Guideline deviations and reporting deficiencies. Several parameters in the MNT not reported. Blind scoring reported for the CA but not MNT. Exposure route used in the MNT is not relevant for human exposure. (see guideline deviations). No indication of pH or osmolality control for the comet assay. Results not reported separately for replicates.
Relevance of study:	Not relevant (Due to guideline deviations and reporting deficiencies).
Klimisch code:	3

End point	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
Bone marrow erythrocyte micronucleus	Mouse	Analytical glyphosate (96%)	2 x 200 mg/kg i.p.	Positive	Erythrocytes scored? TO, SC, IC, RE	Manas et al., 2009 (ASB2012-11892)

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.
[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.
[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

Bolognesi et al. 1997 (Z59299), as described in the previous RAR (2015):

Author(s)	Year	Study title
Bolognesi, C. Bonatti, S.	1997	Genotoxic activity of glyphosate and its technical formulation roundup Journal of Agricultural and Food Chemistry

Degan, P. Gallerani, E. Peluso, M. Rabboni, R. Roggieri, P. Abbondandolo, A.		Volume: 45 Pages: 1957-1962 Z59299

Abstract*
Glyphosate (N-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated in vivo in bone marrow of mice as micronuclei frequency and in vitro in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances *in vivo* and *in vitro*. A weak increment of the genotoxic activity was evident using the technical formulation.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Basic data given, however, the study is performed with methodological and reporting deficiencies (only data without metabolic activation generated in *in vitro* tests, no positive controls included in *in vitro* SCE and *in vivo* experiments, in some experiments only two test substance concentrations tested).
Relevance of study:	Not relevant (Due to methodological and reporting deficiencies data considered to be supplemental information; i.p. exposure route is not relevant for human exposure)
Klimisch code:	3

"Bolognesi et al. (1997, Z59299) reported positive results from a micronucleus test in mouse bone marrow erythrocytes. Either glyphosate a.i. (declared as 99.9 % pure) or a Roundup formulation were administered to Swiss mice once daily by the i.p. route on two consecutive days. Cell samples were harvested at 6 and 24 hours following the final dose. A weak positive effect was observed at total dose levels of 300 mg/kg bw (2 x 150 mg/kg bw/day) after 24 hours for glyphosate and of 450 mg/kg bw (2 x 225 mg/kg bw/day) at both sampling times for Roundup.
Further data in this publication indicated for high purity glyphosate a significant and dose-dependent increase in SCE frequency in human lymphocyte cultures obtained from two female donors from a concentration of 1000 µg/mL onwards. For Roundup, this effect became apparent even at lower concentrations of 100 and 330 µg/mL.
Comment: The outcome of the micronucleus test with glyphosate a.i. is at least surprising since much higher doses of this compound had been tested before and did not reveal indications of clastogenicity (see section B.5.4.2.1 in the monograph). A direct comparison is not possible since the only available test using the i.p. route in which the highest dose of 1000 mg/kg bw proved negative [redacted], 1983, TOX9552369) was performed in rats. The respective study by Rank et al. (1993, Z82234, see above) was conducted in mice but the test material was glyphosate IPA salt and the dose administered was probably too low for meaningful evaluation. However, a number of well-performed micronucleus tests with oral administration to mice is available. Even when the low oral absorption rate of glyphosate (about 30%) is taken into account, the dose levels (up to 5000 mg/kg bw nominal) are much higher than those given by Bolognesi and her co-workers but no convincing evidence of a potential to cause chromosome aberrations in vivo was obtained. It should be emphasized that the increase in the incidence of micronucleated polychromatic erythrocytes as reported in this publication was rather weak only. The test was not performed according to the current OECD guideline. In particular, the number of animals used (three male mice per dose group) was too low since a group size of at least five is recommended. A dose response cannot be assessed since only one dose level was included. The basis for statistical comparison is questionable since it is not clear when the six control animals were sacrificed because only one group mean value was indicated. Due to these

deficiencies, this isolated positive finding is not considered to provide sufficient evidence to contravene the previously obtained negative results regarding the active substance.
The same methodical shortcomings apply to the experiment with the Roundup fomulation. The formulation tested is reported to contain 30.4 % glyphosate acid equivalents. The a.i. is formulated as the IPA salt. Alkyl sulfate surfactant (MON 8080) is also contained (source of information: Monsanto). The weak positive response is in contrast to the beforementioned GLP-like study by Kier et al. (1992, TOX1999-242) in which Roundup® proved negative. However, these two Roundup formulations were not identical since the glyphosate concentrations were nearly the same but the surfactants contained were different making a direct comparison of the study results difficult. Little is known on mutagenicity of alkyle sulfate itself, however, MON 8080 proved negative in the Ames test but was clearly cytotoxic at relatively low concentrations (see section III of this addendum). Some evidence of bone marrow cytotoxicity was obtained with both Roundup products as indicated by a decrease in the ratio between polychromatic and normochromatic erythrocytes. Cytotoxicity could have also an impact on chromosome aberration frequency. An overall, unequivocal conclusion from the experiment of Bolognesi and her group cannot be drawn, however an actual clastogenic response is not very likely. Even if a positive result could be confirmed, it would not be applicable to products containing other surfactants."

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
Micronucleus test in mouse bone marrow; two i.p. administrations with a 24-h interval between; sampling after 6 and 24 h after the final dose	Glyphosate a.i. (99.9% pure) and Roundup (IPA salt with 30.4% glyphosate a.e.; alkyle sulfate surfactant)	0, 300 mg/kg bw (2X150 mg/kg bw/d) for glyphosate; 0, 450 mg/kg bw (2x225 mg/kg bw/d) for Roundup	Weakly positive for glyposate after 24 h and for Roundup at both sampling times. Some evidence of bone marrow cytotoxicity of Roundup.	Supplementary study (methodical deficiencies) revealing an increase in micronucleus frequency, data in contrast to previous results.	Bolognesi et al., 1997 Z59299

Rank et al. 1992 (Z82234), as described in the previous RAR (2015):

"Rank et al. (1993, Z82234) studied the mutagenic potential of the herbicide Roundup and of glyphosate isopropylamine salt in different test systems *in vitro* as well as *in vivo*.
In a micronucleus test in mouse bone marrow erythrocytes following single i.p. administration, Roundup as well as the IPA salt (i.e., a 1:1 mixture of glyphosate technical and isoproplyamine) proved negative up to the highest dose of 200 mg/kg bw. However, with Roundup but not with the glyphosate IPA salt alone, there was evidence of bone marrow cytotoxicity at this top dose level as indicated by a significantly lower percentage of polychromatic erythrocytes.
Comment: According to the publication and to further information submitted by Monsanto, it is assumed that the Roundup formulation used was made of 48 % IPA salt, tallowamine surfactant, and water.
The design of the micronucleus test was also not in compliance with guideline requirements. A direct comparison between results obtained with the IPA salt and Roundup is not feasible since not exactly the same dose levels were used and since there was a difference in sampling time (24 and 48 h post dosing for the IPA experiment versus only at 24 h after administration of Roundup). The negative outcome of previous micronucleus studies with the IPA salt (Rodeo® formulation, Kier et al., 1992, TOX9552376) and with a similar Roundup formulation in mice (Kier et al., 1992, TOX1999-242) was confirmed. The reported weak bone marrow cytotoxicity occurring already after single i.p. administration of 200 mg Roundup/kg bw (amount calculated as the IPA salt to facilitate comparison) may be considered a possible formulation-related effect when the observations in other micronucleus studies (see section I) are taken into consideration."

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
Micronucleus test in mouse bone marrow; single i.p. administration; sampling after 24 or 48 h	Glyphosate IPA salt (1:1 mixture) and Roundup (48% glyphosate IPA; tallowamine surfactant)	0, 100, 150, 200 mg/kg bw (glyphosate IPA); 0, 133, 200 mg/kg bw (Roundup, calculated as IPA salt)	Negative. Indication of dose-related bone marrow cytotoxicity with the Roundup formulation but not with glyphosate IPA.	Supplementary study confirming previous results.	Rank et al., 1993 Z82234

Chruscielska et al. 2000 (ASB2013-9830), as described in the previous RAR (2015):

End point	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vivo Chromosome Effects—Mammalian Systems						
Bone marrow erythrocyte micronucleus	Mouse	Glyphosate	300 mg/kg i.p. Perzocyd 10 SL formulation	Negative Negative	DL, TO, SC, IM, RE DL, TO, SC, IM, RE	Chruscielska et al., 2000, (ASB2013-9830)

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.
[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.
[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

***In vivo* DNA damage**

Bolognesi et al. 1997 (Z59299), as described in the previous RAR (2015):

Author(s)	Year	Study title
Bolognesi, C. Bonatti, S. Degan, P. Gallerani, E. Peluso, M. Rabboni, R. Roggieri, P. Abbondandolo, A.	1997	Genotoxic activity of glyphosate and its technical formulation roundup Journal of Agricultural and Food Chemistry Volume: 45 Pages: 1957-1962 Z59299

Abstract*
Glyphosate (N-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated in vivo in bone marrow of mice as micronuclei frequency and in vitro in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances *in vivo* and *in vitro*. A weak increment of the genotoxic activity was evident using the technical formulation.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Basic data given, however, the study is performed with methodological and reporting deficiencies (only data without metabolic activation generated in *in vitro* tests, no positive controls

	included in *in vitro* SCE and *in vivo* experiments, in some experiments only two test substance concentrations tested).
Relevance of study:	Not relevant (Due to methodological and reporting deficiencies data considered to be supplemental information; i.p. exposure route is not relevant for human exposure)
Klimisch code:	3

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
Alkaline elution assay for DNA single-strand breaks and formation of alkali labile sites in DNA obtained from liver and kidneys of mice following single i.p.administration	Glyphosate a.i. (99.9% pure) and Roundup (IPA salt with 30.4% glyphosate a.e.; alkyle sulfate surfactant)	0, 300 (glyphosate a.i.), 900 (Roundup) mg/kg bw; sampling after 4 and 24 h	Weakly positive after 4 h in both organs suggesting possible transient DNA damage.	Supplementary study (methodical deficiencies). Biological significance equivocal. Results in contrast to the negative outcome of the UDS assay. Effects might be also due to toxicity.	Bolognesi et al., 1997 Z59299

Peluso et al., 1998 (TOX1999-318), as described in the previous RAR (2015):

Author(s)	Year	Study title
Peluso, M. Munnia, A. Bolognesi, C. Parodi, S.	1998	^{32}P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. Environmental and Molecular Mutagenesis Volume: 31 Number: 4 Pages: 55-59 TOX1999-318

Abstract*
Roundup is a postemergence herbicide acting on the synthesis of amino acids and other important endogenous chemicals in plants. Roundup is commonly used in agriculture, forestry, and nurseries for the control or destruction of most herbaceous plants. The present study shows that Roundup is able to induce a dose-dependent formation of DNA adducts in the kidneys and liver of mice. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 +/- 0.1 (SE) and 1.7 +/- 0.1 (SE) adducts/10(8) nucleotides, respectively. The Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate, but to another, unknown component of the herbicide mixture. Additional experiments are needed to identify the chemical specie(s) of Roundup mixture involved in DNA adduct formation. Findings of this study may help to protect agricultural workers from health hazards and provide a basis for risk assessment.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not Reliable
Comment:	A non-guideline study with confounding results based on testing a surfactant containing formulation. Reporting deficiencies (statistical methods). Toxic surfactant effects subsequently verified in Heydens et al. (2008, ASB2012-11845) reporting the same study type with a glyphosate formulated product and an appropriate control; formulation blank without glyphosate.
Relevance of study:	Not relevant (i.p. administration of high doses of a surfactant containing formulation a relevant exposure scanario for human risk assessments. In addition, the DNA adducts observed were not related to the active ingredient (isopropylammonium salt of glyphosate), but to another, unknown component of the herbicide mixture.)

Klimisch code: 3

Test method/ test system	Test material	Dose levels/ Dose range	Results	Remarks	Reference
Measuring of DNA adducts by means of ^{32}P-postla-beling technique in the liver and kidney of mice following single i.p. administration	Glyphosate IPA salt and Roundup (IPA salt with 30.4% glyphosate a.e.; alkyle sulfate surfactant)	0, 130, 270 mg/kg bw (glyphosate IPA); 0, 400, 500, 600 mg/kg bw (Roundup)	Weak dose-related increase in adducts with Roundup; no adducts seen with the IPA salt alone and in the control group.	Indication of possible DNA damage, however, biolo-gical significance of this finding equivocal. Further characterization of ad-ducts needed. Toxicity not adressed. However, non-mutagenic toxic effects can also cause DNA adducts.	Peluso et al., 1998 TOX1999-318

"A possible impact on the DNA was also investigated by Bolognesi et al. (1997, Z59299) in further experiments. A transient but significant effect towards DNA damage in liver and kidney was noted in the alkaline elution assay after glyphosate (300 mg/kg bw) or Roundup (900 mg/kg bw) had been administered once by the i.p. route to mice. This assay may indicate the induction of DNA single-strand breaks and alkali labile sites. A test for DNA oxidative damage suggested glyphosate and the formulation Roundup to stimulate oxidative metabolism in the liver (glyphosate) or in the kidney (Roundup) at 24 hours after application.
In a subsequent study from the same institute (Peluso et al., 1998, TOX1999-318), a low incidence of DNA adducts was found by means of the very sensitive ^{32}P-postlabeling technique in the liver and kidney of mice following single intraperitoneal administration of Roundup. All tested concentrations (400, 500 and 600 mg Roundup/kg bw, corresponding to 122, 152, and 182 mg glyphosate salt/kg bw) caused DNA adducts in both organs. A dose response was to be seen. In contrast, treatment with the vehicle (i.e., a DMSO/olive oil mixture) and with doses of 130 and 270 mg glyphosate IPA salt/kg bw did not result in DNA adduct formation.

<u>Comment:</u> The data from the tests for DNA damage and stimulation of oxidative metabolism (Bolognesi et al., 1997, Z59299) are hardly to interpret since the results are given in summary figures only which are based on pooled individual data. There are reporting inconsistencies, e.g. it is not clear how many animals were actually used for testing. A positive control substance was not included. Taking into account that glyphosate proved negative in the UDS assay which is generally accepted to indicate a more frequent occurrence of DNA damage and repair (see section B.5.4.1.3 in the monograph), the published findings are not considered to provide convincing evidence of an interaction with the DNA. Positive results in the alkaline elution assay may also occur as a result of toxic but not-mutagenic effects. Stimulation of oxidative metabolism is not a sign of mutagenicity but may elucidate a possible mechanism behind toxic effects.
The results of Peluso (1998, TOX1999-318) and his group suggest a direct effect on the DNA. It has been shown that the observed effects were related to administration of the formulation only but not to glyphosate IPA salt. Biological significance of the results is equivocal. Generally, it is questionable whether findings after i.p. administration can be applied to more realistic exposure conditions. Of course, the occurrence of such effects also after oral intake would be much more relevant for human health evaluation. Furthermore, some deficiencies of this study make a definitive assessment difficult. It is rather equivocal what a low incidence of DNA adducts per animal as compared to no adducts in the control group actually means since a positive control substance was not included. The degree of variation between the animals is not known because only mean values for the groups comprizing of 3 to 6 mice were reported and individual values are not given but would be helpful for interpretation of the results. Another point of concern is the lacking information on toxicity. At least with Roundup, one could expect marked general toxicity when the observations reported from the micronucleus tests (see section I of this addendum) and from the acute intraperitonal toxicity studies (see section B.5.2.4 in the monograph) were taken into account. It is known that DNA adducts may be formed not only as a result of direct interaction of cellular DNA with chemicals but also occur naturally or can be even related to a treatment-dependent increase in

endogenous metabolites. Thus, further characterisation of these adducts and clarification of their nature would be desirable."

B.6.4.4.17. *Public literature referenced in RAC opinion (2017) on oxidative stress*

In volume 1, section 2.6.4.1 reference is also made to the RAC opinion on glyphosate from March 2017 in which several studies on oxidative stress were discussed. The applicant did not submit these studies, as the studies were published over 10 years ago which is outside the scope of the current public literature search and as the studies were considered not reliable/relevant or of limited reliability/relevance during the previous assessment. For completeness, RMS has included the summaries and evaluations of these ~~two~~ studies from the previous RAR (2015) and these are shown below. RMS has **not** re-evaluated this information as these studies were considered not reliable/relevant or of limited reliability/relevance during the previous assessment.

Bolognesi et al. 1997 (Z59299), as described in the previous RAR (2015):

Author(s)	Year	Study title
Bolognesi, C. Bonatti, S. Degan, P. Gallerani, E. Peluso, M. Rabboni, R. Roggieri, P. Abbondandolo, A.	1997	Genotoxic activity of glyphosate and its technical formulation roundup Journal of Agricultural and Food Chemistry Volume: 45 Pages: 1957-1962 Z59299

Abstract*
Glyphosate (N-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated in vivo in bone marrow of mice as micronuclei frequency and in vitro in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances *in vivo* and *in vitro*. A weak increment of the genotoxic activity was evident using the technical formulation.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Basic data given, however, the study is performed with methodological and reporting deficiencies (only data without metabolic activation generated in *in vitro* tests, no positive controls included in *in vitro* SCE and *in vivo* experiments, in some experiments only two test substance concentrations tested).
Relevance of study:	Not relevant (Due to methodological and reporting deficiencies data considered to be supplemental information; i.p. exposure route is not relevant for human exposure)
Klimisch code:	3

Peluso et al. 1998 (TOX1999-318), as described in the previous RAR (2015):

Author(s)	Year	Study title
Peluso, M. Munnia, A. Bolognesi, C. Parodi, S.	1998	^{32}P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. Environmental and Molecular Mutagenesis Volume: 31

		Number: 4 Pages: 55-59 TOX1999-318

Abstract*
Roundup is a postemergence herbicide acting on the synthesis of amino acids and other important endogenous chemicals in plants. Roundup is commonly used in agriculture, forestry, and nurseries for the control or destruction of most herbaceous plants. The present study shows that Roundup is able to induce a dose-dependent formation of DNA adducts in the kidneys and liver of mice. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 +/- 0.1 (SE) and 1.7 +/- 0.1 (SE) adducts/10(8) nucleotides, respectively. The Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate, but to another, unknown component of the herbicide mixture. Additional experiments are needed to identify the chemical specie(s) of Roundup mixture involved in DNA adduct formation. Findings of this study may help to protect agricultural workers from health hazards and provide a basis for risk assessment.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not Reliable
Comment:	A non-guideline study with confounding results based on testing a surfactant containing formulation. Reporting deficiencies (statistical methods). Toxic surfactant effects subsequently verified in Heydens et al. (2008, ASB2012-11845) reporting the same study type with a glyphosate formulated product and an appropriate control; formulation blank without glyphosate.
Relevance of study:	Not relevant (i.p. administration of high doses of a surfactant containing formulation a relevant exposure scanario for human risk assessments. In addition, the DNA adducts observed were not related to the active ingredient (isopropylammonium salt of glyphosate), but to another, unknown component of the herbicide mixture.)
Klimisch code:	3

Mladinic et al. 2009 (ASB2012-11906), as described in the previous RAR (2015):

Author(s)	Year	Study title
Mladinic, M. Berend, S. Vrdoljak, A.L. Kopjar, N. Radic, B. Zeljezic, D.	2009	Evaluation of Genome Damage and Its Relation to Oxidative Stress Induced by Glyphosate in Human Lymphocytes *in Vitro* Environmental and Molecular Mutagenesis Volume: 50 Number: 9 Pages: 800-807 ASB2012-11906

Abstract*
In the present study we evaluated the genotoxic and oxidative potential of glyphosate on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure. Testing was done with and without metabolic activation (S9). Ferric-reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and the hOGG1 modified comet assay were used to measure glyphosate's oxidative potential and its impact on DNA. Genotoxicity was evaluated by alkaline comet and analysis of micronuclei and other nuclear instabilities applying centromere probes. The alkaline comet assay showed significantly increased tail length (20.39 μm) and intensity (2.19 %) for 580 μg/mL, and increased tail intensity (1.88 %) at 92.8 μg/mL, compared to control values of 18.15 μm for tail length and 1.14 % for tail intensity. With S9, tail length was significantly increased for all concentrations tested: 3.5, 92.8, and 580 μg/mL. Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91 μg/mL with S9 and 580 μg/mL without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5 μg/mL and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580

μg/mL. FRAP values slightly increased only at 580 μg/mL regardless of metabolic activation, while TBARS values increased significantly. Since for any of the assays applied, no clear dose-dependent effect was observed, it indicates that glyphosate in concentrations relevant to human exposure do not pose significant health risk.
* Quoted from article

Klimisch evaluation

Reliability of study:	Reliable with restrictions
Comment:	Non-GLP, non-guideline *in vitro* study, meeting scientific principles
Relevance of study:	Relevant with restrictions (Assessment of Genotoxicity *in vitro* at concentrations relevant to human exposure levels; authors state that no clear dose-dependent effect was observed, and results indicate that glyphosate in concentrations relevant to human exposure do not pose significant health risk.
Klimisch code:	2

Manas et al. 2009 (ASB2012-11892), as described in the previous RAR (2015):

Author(s)	Year	Study title
Manas, F. Peralta, L. Raviolo, J. Garcia Ovando, H. Weyers, A. Ugnia, L. Gonzalez Cid, M. Larripa, I. Gorla, N.	2009	Genotoxicity of glyphosate assessed by the comet assay and cytogenic tests Environmental Toxicology and Pharmacology Volume: 28 Pages: 37-41 ASB2012-11892

Abstract*
It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42±1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p < 0.01$) with glyphosate at a range concentration from 3.00 to 7.50mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20–6.00mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT)was evaluated in mice at three doses rendering statistical significant increases at 400 mg/kg (13.0±3.08 micronucleated erythrocytes/1000 cells, $p < 0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Guideline deviations and reporting deficiencies. Several parameters in the MNT not reported. Blind scoring reported for the CA but not MNT. Exposure route used in the MNT is not relevant for human exposure. (see guideline deviations). No indication of pH or osmolality control for the comet assay. Results not reported separately for replicates.
Relevance of study:	Not relevant (Due to guideline deviations and reporting deficiencies).
Klimisch code:	3

Astiz et al. 2009 (ASB2012-11549), as described in the previous RAR (2015):

Author(s)	Year	Study title
Astiz, M. de Alaniz, M.J. Marra, C.A.	2009	Effect of pesticides on cell survival in liver and brain rat tissues Ecotoxicology and Environmental Safety Volume: 72, Pages: 2025-2032, ASB2012-11549

Abstract*
Pesticides are the main environmental factor associated with the etiology of human neurodegenerative disorders such as Parkinson's disease. Our laboratory has previously demonstrated that the treatment of rats with low doses of dimethoate, zineb or glyphosate alone or in combination induces oxidative stress (OS) in liver and brain. The aim of the present work was to investigate if the pesticide-induced OS was able to affect brain and liver cell survival. The treatment of Wistar rats with the pesticides (i.p. 1/250 LD50, three times a week for 5 weeks) caused loss of mitochondrial transmembrane potential and cardiolipin content, especially in substantia nigra (SN), with a concomitant increase of fatty acid peroxidation. The activation of calpain apoptotic cascade (instead of the caspase-dependent pathway) would be responsible for the DNA fragmentation pattern observed.
Thus, these results may contribute to understand the effect(s) of chronic and simultaneous exposure to pesticides on cell survival.
* Quoted from article

Klimisch evaluation

Reliability of study:	Not reliable
Comment:	Unsuitable test system (i.p exposure route is not relevant for human exposure). No information on purities of test substances used. Small group size (4 males/dose group), reporting deficiencies
Relevance of study:	Not relevant (intraperitoneal injection is a non-relevant route of exposure for humans)
Klimisch code:	3

Additional comments
This non-guideline study utilized very small group numbers (4 rats/group) and therefore is not sufficiently robust to appropriately identify changes attributable to the test material administration.
The test materials are not well described, without indication of whether a glyphosate salt form or acid was used and purity was not reported.
The publication focuses on the post necropsy data analysis and reporting. Data on animal husbandry, clinical observations, feed and water intake, weekly body weight were not reported, but the authors note there were no adverse observations.
No statistically significant effects were noted for liver endpoints, yet the liver is in close proximity to test material administration via intraperitoneal injection.
Statistically significant effects were noted for brain tissue endpoints in the substantia nigra and cerebral cortex. However, there is a lack of biological plausibility for brain exposures to glyphosate, given the necessity to pass the blood-brain barrier and the known rapid elimination kinetics of this polar molecule via urine.

B.6.4.4.18. *Genotoxicity – public literature, study 15 (study for which the RMS requested a summary in order to further justify the categorization)*

Data point:	CA 5.4
Report author	Alvarez-Moya C. *et al.*
Report year	2014
Report title	Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms.
Document source	Genetics and molecular biology (2014),Vol. 37, No. 1, pp. 105-110
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)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

There is considerable controversy with regard to the genotoxicity of glyphosate, with some reports stating that this compound is non-toxic for fish, birds and mammals. The present study was performed to investigate the genotoxicity of glyphosate isopropylamine salt (batch: 09816 PE, purity: 96%) in different organisms. A comet assay was performed in human lymphocytes, erythrocytes of *Oreochromis niloticus* and staminal nuclei of *Tradescantia in vitro* and *in vivo*. The present summary focuses on the experiment in human lymphocytes only.

The cells were exposed to glyphosate concentrations in the range of 0.7-700 µM for 20 hours at 25 °C. Corresponding untreated and positive controls (5 mM N-nitrosodiethyleneamine) were included in each experiment. Following exposure, electrophoresis was performed and approximately 100 cells or nuclei per condition were evaluated for their migration (rail length).

There was a statistically significant and concentration-dependent increase in the migration (tail length) of human lymphocyte DNA in the comet assay after treatment with glyphosate when compared to untreated control cells. For the positive control, a statistically significant increase in tail length was noted, demonstrating the sensitivity of the test.

The authors compared the data from a study of the direct effect of glyphosate in occupationally exposed humans with the effect of direct exposure of human lymphocytes to glyphosate observed here. The tail length was very similar *in vivo* and *in vitro*, and there was a positive relationship between genotoxicity and glyphosate concentration.

Under the conditions of the study, glyphosate is considered to be genotoxic in human lymphocytes.

Materials and methods

Test Material:	Glyphosate (N-(phosphonomethyl)-glycine
Origin:	Aldrich Chemical Co. (St. Louis, Missouri, USA)
Lot/Batch number:	09816 PE
Purity:	96%
CAS#:	1071-83-6
Vehicle:	Not reported
Positive control:	N-nitrosodiethylamine (NDEA), 5mM
Test Organisms:	1. Human lymphocytes: 2. *Tradescantia* (clone 4430) staminal nuclei[12] 3. *Oreochromis niloticus* erythrocytes[1]

Preparation of human lymphocytes
Cells from 8 individuals were used. For the preparation of human lymphocytes, peripheral blood samples were obtained from a finger puncture in young students who initially responded to a questionnaire to rule out exposure to genotoxic agents. Individuals on medical treatment, smokers, drug users and inhabitants of the contaminated area of Guadalajara, Jalisco, Mexico were excluded from the study. Each blood sample was placed in a test tube containing 3 mL of phosphate-buffered saline (PBS; 160 mM NaCl, 8 mM Na_2HPO_4, 4 mM NaH_2PO_4 and 50 mM EDTA; pH 7) and immediately centrifuged at 3000 rpm for 5 min. The supernatant was removed and the pellet was re-suspended in phosphate buffer and immediately stored at 4 °C until used.

[12] For this summary, only the experiment in human peripheral lymphocytes is reported and considered relevant for risk assessment.

Cell viability was assessed with the Trypan Blue test using 20 mL of peripheral blood lymphocytes. The mean percentage viability for each group was >89%.

Slide preparation and treatment
Harvested cells were used for slide preparation, followed by treatment with glyphosate isopropylamine salt. The slides were exposed to glyphosate concentrations of 0.7, 7, 70 and 700 µM for 20 hours at 25 °C. Slides which remained untreated were used as negative control and slides treated with 5 mM N-nitrosodiethylemaine were used as positive control. After treatment, the slides were washed and stored at 4 °C. The entire experiment was repeated twice.

Comet assay
The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% sodium lauryl sarcosine, 1% Triton X-100 and 10% DMSO, pH 10) for 20 h at 4 °C to ensure nuclear lysis and then placed in a horizontal electrophoresis system with a high pH buffer (30 mM NaOH, 1 mM Na_2EDTA, pH 13) for 45 minutes to allow DNA unfolding before electrophoresis for 20 min at 1.0 V/cm with an amperage of ~300 mA. The whole procedure was conducted under yellow light to prevent additional changes to the DNA.

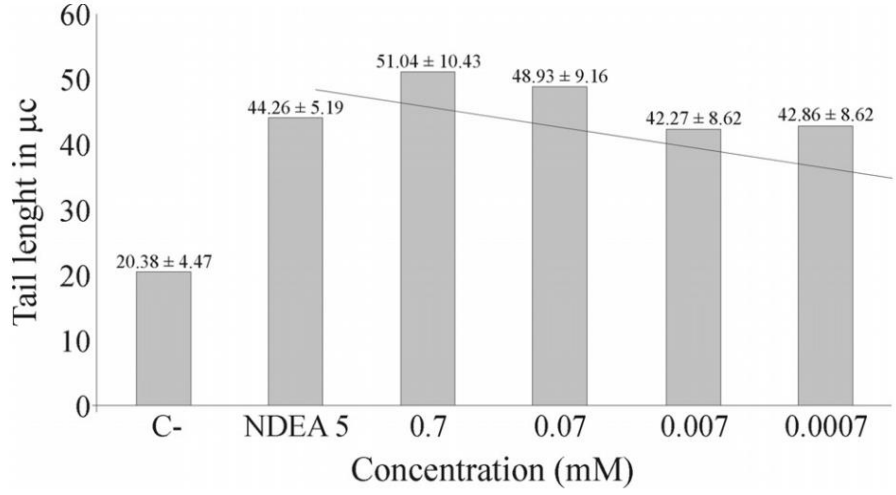
After electrophoresis, the slides were gently washed to remove the alkaline solution and then immersed in neutralization buffer (0.4 M Tris base, pH 7.5) for 5 minutes. The gels were stained with ethidium bromide (100 µL at 20 µg/mL) for 3 minutes and then rinsed three times with distilled water. The preparation was subsequently covered with a coverslip and the slides were examined by fluorescence microscopy using a light microscope equipped with a 515-560 nm excitation filter. Cells were observed at 10X magnification and tail length during migration was determined by using Comet assay software based on published protocols. Approximately 50 cells or nuclei per slide and two slides for each experimental point and controls were evaluated.

Data analysis
The results were expressed as the mean ± SD and were analysed by one-way analysis of variance (ANOVA) using the CoStat program. All experimental groups were compared with the corresponding negative control using the Dunnett test. A value of $p \leq 0.05$ indicated significance.

Results

Comet assay of human lymphocytes in vitro
There was a statistically significant and concentration-dependent increase in the migration (tail length) of human lymphocyte DNA in the comet assay after treatment with glyphosate when compared to untreated control cells. For the positive control, a statistically significant increase in tail length was noted (Figure 1), demonstrating the sensitivity of the test.

Figure 1. Tail length in human lymphocytes exposed to different concentrations of isopropylamine glyphosate. The diagonal line indicates the relationship between glyphosate concentration and tail length in micrometers (µc). C-, negative control; NDEA 5, 5 mM N-nitrosodiethylamine. The values above the columns are the mean ± SD (n = 8).



In this study, the comet assay clearly showed that glyphosate was genotoxic in the cells examined. Since the *in vivo* effect of glyphosate in humans was not examined in this work, the authors compared the data from a study of the direct effect of glyphosate in occupationally exposed humans (Paz-y-Miño et al., 2007) with the effect of direct exposure of human lymphocytes to glyphosate observed here. As shown in Table 1, glyphosate was genotoxic in both studies. The tail length was very similar in both cases, and there was a positive relationship between genotoxicity and glyphosate concentration/dose.

Table 1. Comparison between human lymphocytes from persons occupationally exposed to glyphosate and human lymphocytes exposed directly to various concentrations of the compound.

Study *in vivo* (Paz-y-Miño *et al.*, 2007)	mM	Tail length (μc)
Individuals exposed		35.5 ± 6.4
Individuals not exposed		25.9 ± 0.6
Study *in vitro* (present study)		
Lymphocytes exposed	0.7	51.0 ± 10.4
	0.07	48.9 ± 9.2
	0.007	42.3 ± 8.6
	0.0007	42.9 ± 8.6
Lymphocytes not exposed		20.4 ± 4.1

The values are the mean ± SD (n = 8).

Conclusion

Based on the experimental findings and under the conditions of the test, glyphosate induced a dose-dependent increase in tail length in the comet assay in human lymphocytes and is therefore considered to be genotoxic.

References

Paz-y-Miño C, Sánchez ME, Arévalo M, Muñoz MJ, Witte T, Oleas G, and Leone PE (2007) Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genet Mol Biol 30:456-460.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Mechanistic study without clear relevance for the risk assessment.

Further points for clarification:
This paper describes the effects of glyphosate on human lymphocytes, erythrocytes of *Oreochromis niloticus* and staminal nuclei of *Tradescantia*. In the context of the human health risk assessment, only the human lymphocyte data have been considered. Glyphosate was found to induce migration (tail length) of human lymphocyte DNA, thereby identifying the substance as genotoxic.

There are several deficiencies in the reporting of methods and results, which adversely affect the reliability of the data and results reported. No information is provided on the solvent used to prepare glyphosate concentrations and the negative controls appear to be untreated cells and not a solvent control. It is unclear if the negative controls were treated in the same way as the glyphosate treated cells (i.e. no exposure to solvent only and unknown if cells were held at 25 °C for 20 hours). Similarly, insufficient detail is provided for the positive control treatments. Cell viability is reported as being determined by trypan blue exclusion, however, it is not stated when this was determined or if a post-treatment viability/cytotoxicity assessment was conducted. In addition, the time the lymphocytes were held at 4 °C before and after treatment has not been reported. Consequently, it is unknown to what degree toxicity may have been responsible for the findings. Although statistical methods are described, the statistical results have not been reported in detail. Furthermore, the Results section indicates additional comparisons (comparison of glyphosate concentration amongst themselves and

comparisons of glyphosate concentrations to positive controls) not described in the methods. The Methods indicate blood from 8 donors was tested and that the entire experiment was repeated twice. However, the Results graph presents mean ± SD for an n = 8; it is unknown if the results were reproducible across the two experiments. There is no information regarding how comet assays were selected for image analysis, for example, it is unknown if cells with a cloud/hedgehog cell morphology were identified and or scored as comets. No historical negative or positive control data have been reported.

In this paper, glyphosate was tested in human lymphocytes at concentrations ranging from 0.7 to 700 µM. Exposures were performed for 20 hours. The authors report that all concentrations induced a statistically significant increase in tail length compared to the negative control and that was generally proportional to the concentration of glyphosate. However, a closer examination of the graph representing the data suggests a lack of any linear trend and there is considerable overlap of the standard deviations between the four treated groups. The group mean tail lengths reported for glyphosate groups range from 42.27 ± 8.62 to 51.04 ± 10.43. In addition, the data indicate that the degree of DNA damage is similar to that induced by the positive control, the potent genotoxin N-nitrosodiethylamine, which was tested at 5 mM. No data for lymphocyte cultures exposed to solvent only for 20 hours at 25 °C is provided and statistical comparison have been made to untreated lymphocytes. The observation that a similar degree of DNA damage is induced by glyphosate concentrations covering 4 orders of magnitude (the highest of which is more than 7x lower than the positive control concentration used) would suggest the findings are due to methodology effects rather than glyphosate specific genotoxicity. The authors claim that glyphosate is genotoxic under these conditions cannot be substantiated without presentation of data from an appropriately treated solvent (negative) control group, detailed information regarding post-treatment cytotoxicity in all cultures and a clear description of the cell-selection criteria for analysis.

The authors compare the *in vitro* data to biomonitoring data from humans occupationally exposed to glyphosate (reported in a separate publication) and claim glyphosate was genotoxic in both studies, with similar tail lengths in both studies and a positive relationship between genotoxicity and glyphosate concentration/dose. This comparison would not appear to be relevant given the *in vitro* data were generated from lymphocytes directly exposed to glyphosate for 20 hours in the absence of any exogenous metabolizing system compared to occupational human exposures.

This publication is considered relevant for the risk assessment of glyphosate (5.4.1 case B) and reliable with restrictions because no cytotoxicity assay was conducted to optimise the range of test concentrations, lack of adequate negative control, lack of a true solvent control group, no HCD and poor reporting of statistics. Therefore, the publication is considered to provide supplementary information.

Assessment and conclusion by RMS:

In this study human lymphocytes, erythrocytes of Tilapia fish and nuclei of *Tradescantia* (4430) plants were exposed to 0.7, 7, 70 and 700 µM glyphosate isopropylamine salt *in vitro* to investigate DNA damage. Additionally, eight fish per dose were exposed to different concentrations of Roundup® and approximately 10 *Tradescantia* (4430) flowers were immersed for 3 h in 250 mL of glyphosate isopropylamine salt. The purity and lot number were provided for the glyphosate used, however, the composition of the tested Roundup formulation was not provided.
A mean percentage viability of >89% for each group of the blood lymphocytes and erythrocytes is reported. No results of cell viability are reported for the nuclei of *Tradescantia* (4430) plants nor is there information on the bioavailability of the tested compounds *in vivo* for both the Tilapia fish and *Tradescantia* (4430) plants.
The cells of the different organisms were analysed for DNA breaks using the comet assay system.

The test substance stability and test concentration were not analytically verified, the age and sex of the fish was not reported, no general clinical observations of the animals were made besides no reported mortality, only 100 cells or nuclei per dose were evaluated instead of 150 and no information on cell scoring and the identification of hedgehog cells is reported. Both *in vitro* and *in vivo* exposure to glyphosate resulted in an increased tail length compared to the control, with a stronger effect seen *in vitro*. The results on fish and plants were not described by the applicant in the summary which is considered acceptable as the relevance to human exposure is unclear (species differences, difference in exposure route and level of exposure).

The study has several limitations, including: negative controls were untreated instead of treatment with a vehicle control, no historical data were provided, no details are given on the human donors, it is unclear if the slides were scored “blinded”, only 50 cells or nuclei per slide were scored (with two slides for each experiment)

whereas OECD 489 indicates that at least 150 cells should be scored for each sample. Furthermore, it is not clear if cytotoxicity was assessed after exposure treatment, whereas it is known that target tissue toxicity may also result in increases in DNA migration. The study as described in this article does not meet the acceptability criteria as defined in OECD489.
Based on these limitations, this study is considered to be reliable with restrictions.

B.6.4.4.19. *Genotoxicity – public literature, study 16 (study for which the RMS requested a summary in order to further justify the categorization)*

Data point:	CA 5.4
Report author	Rodrigues H. G. *et al.*
Report year	2011
Report title	Effects of roundup pesticide on the stability of human erythrocyte membranes and micronuclei frequency in bone marrow cells of Swiss mice.
Document source	Open Biology Journal (2011), Vol. 4, pp. 54
Guidelines followed in study	None. The micronucleus assay performed in the study was conducted similarly to OECD guideline 474 (2016).
Deviations from current test guideline OECD 474 (2016)	None stated
GLP/Officially recognised testing facilities	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability: as provided in the AIR5 dossier (KCA 9)	Classified as relevant but supplementary (EFSA GD Point 5.4.1 - relevance category B)

2. Full summary of the study according to OECD format

In the first part of the study, the effects of the glyphosate formulation Roundup (batch and purity not reported) on the membrane of human erythrocytes were investigated. Blood samples were collected from 8 human volunteers and investigated for their stability and lysis when treated with Roundup. The concentrations selected for the study were 5-40 mL Roundup/100 mL water, corresponding to the concentration range recommended by the manufacturer. At the concentration limit recommended for agricultural purposes, Roundup promoted 100% of haemolysis. A Roundup concentration of 31.91 ± 3.86 µg/dL caused 50% hemolysis in human erythrocytes. The values obtained for human blood samples collected with EDTA were not significantly different from those obtained for samples collected with heparin. However, the lysis curves presented lower absorbance values at 540 nm in the presence of blood collected with EDTA in relation to that collected with heparin, probably due to haemoglobin precipitation with EDTA. This first experimental section of the study is not part of this summary.

In the second part of the study, a micronucleus test in bone marrow was performed in Swiss mice. Groups of mice were administered an intraperitoneal injection of 0.148, 0.754 or 1.28 mg/kg bw Roundup (batch and purity not reported), vehicle (sterile saline) or positive control (250 mg/kg bw cyclophosphamide). 24 hours following treatment, the animals were sacrificed, bone marrow smears were prepared and erythrocytes were screened for the presence of micronuclei.

For animals of the mid (0.754 mg/kg bw) and high dose level (1.28 mg/kg bw), there was a statistically significant increase in the frequency of micronucleated erythrocytes when compared to control animals. Frequencies for micronucleated cells of the positive control were in the expected range.

Under the conditions of the test, Roundup was found to induce micronuclei in the bone marrow of Swiss mice when tested at concentrations recommended for agricultural use.

Materials and methods

Test Material:	Roundup
Origin:	Not specified
Lot/Batch number:	Not reported

Purity:	Not reported
CAS#:	Not reported
Vehicle:	Sterile saline
Positive control:	Cyclophosphamide, 250 mg/kg bw
Test Animals:	
Species	Mouse
Strain	Swiss
Sex:	Not specified
Age at treatment	7 – 12 weeks
Source	Iquego (Goiânia, Goiás, Brazil)
Housing	In plastic cages.
Diet	*ad libitum*
Water	*ad libitum*
Environmental conditions	Temperature: 26 ± 2 °C Photoperiod: 12 hours light / 12 hours dark

Animal assignment and treatment
The test item was dissolved in sterile saline and administered by intraperitoneal injection to rats at dose levels of 0.148, 0.754 or 1.28 mg/kg bw Roundup. Dose levels were chosen in a range covering the range recommended for use in agriculture (5-40 µg/100 mL water).

A group of negative control animals received the vehicle (sterile saline) and a group of positive control animals was treated with 250 mg/kg bw cyclophosphamide. The animals were sacrificed by cervical dislocation about 24 hours post-treatment.

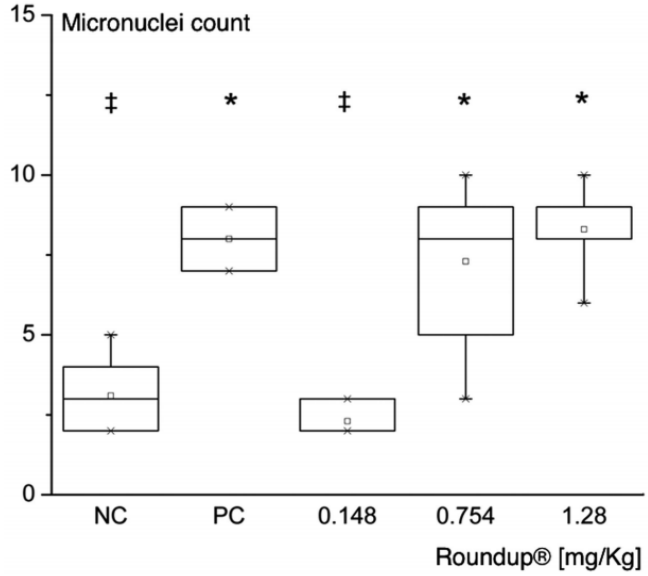
Slide preparation
After sacrifice, the bone marrow of both femurs was collected with the aid of a 1 mL syringe filled with saline. Bone marrow cells were flushed out and collected in a saline-filled test tube. The cell suspension was centrifuged for 5 minutes at 1300 *g*, discarding the supernatant, and retaining a volume of 0.5 mL in the tube for later resuspension and homogenization of the cell precipitate. From the resulting suspension, a small drop was removed and placed in one extremity of the blade for the performance of smears in duplicate. The smears were stained with Leishman's stain after drying, and the slides were dried at room temperature.

Statistics
The comparisons of means between groups were performed by analysis of variance (ANOVA), with $p < 0.05$ indicating statistically significant differences.

Results

There was a statistically significant increase in the frequency of micronucleated erythrocytes in Roundup-treated mice when compared to the solvent control animals at the mid (0.754 mg/kg bw) and the high dose level (1.28 mg/kg bw), which were comparable to those of the positive control. At 0.148 mg/kg bw, no induction of micronuclei was observed.

Figure 1. Frequency of micronuclei in the bone marrow of Swiss mice. NC: negative control; PC: positive control. * and ‡ indicate a statistically significant difference (Tukey test) in relation to NC and PC, respectively.



Conclusion

Under the conditions of the test, Roundup increased the frequency of micronuclei in the bone marrow of Swiss mice when tested at concentrations recommended for agricultural use.

3. Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Substance identification is missing, the study is lacking statistically and, moreover, a mixed study design has been presented where the micronuclei frequency had been investigated in mice after i.p. injection.

The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 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. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Further points for clarification:
In the first part of the study, the authors demonstrated that Roundup, when used at concentrations recommended for agricultural use, promoted 100% of hemolysis in human erythrocytes. The concentration producing 50% hemolysis (D_{50}) was 31.91 ± 3.86 µg/dL and values were comparable for blood samples collected with EDTA and with heparin. However, the first part of the study is not considered in this summary.

In the second part of this study, a micronucleus test in Swiss mice was conducted. The test was performed similar to OECD guideline 474 but with a number of major deviations. There was no information on the number of animals used for the study and the sex of the animals was not indicated. Further, no information on the substance identity (CAS, purity and batch) were provided. No historical control data were reported for the solvent and positive control-treated animals. The test item was administered by intraperitoneal injection, which does not represent a route of exposure relevant to humans. Dose levels selected for the study corresponded to those recommended for agricultural use by the manufacturer of the product, but the route of application is not recommended by the current OECD guideline 474 (2016). No evaluation of toxicity was performed. The PCE/NCE ratio was not determined and no information on general clinical observations of the animals was reported. However, as a positive effect on micronucleus induction was observed, bone marrow exposure of the animals was evident. No acceptance and evaluation criteria were reported. There was no information regarding the evaluation of slides, no information on the number of polychromic or normochromic erythrocytes investigated per animal. In addition, no numeric values were reported in the results part.

Due to the major deviations when compared to current guidelines, the study is considered relevant but to provide supplementary information only (5.4.1 case b).

Assessment and conclusion by RMS:

In this study the effect of Roundup, not glyphosate alone, on the stability of human erythrocyte membranes was evaluated by spectrophotometry and light microscopy, using blood samples from 8 human volunteers collected with EDTA or heparin as anticoagulant agents, as well as the genotoxicity on the bone marrow of Swiss mice. The analysis showed 50% of lysis of human erythrocytes at Roundup concentrations of 31.91 ± 3.86 µL/dL. Above this point the erythrocytes undergo volume contraction, deformation and lysis. In mice, an increase in the occurrence of micronuclei is reported for the two highest doses of the exposed groups (mice were exposed to 0.148 mg/kg bw, 0.754 mg/kg bw and 1.27 mg/kg bw of Roundup).

The study is considered unreliable by the RMS considering the large number of deviations from OECD 474 which are reported by the applicant above. In addition to the deviations already mentioned, the housing temperature of the animals was slightly higher than the desired range and the number of cells that were scored is not reported. Moreover, the study is carried out with a formulation of glyphosate, thus effects caused by co-formulants cannot be excluded. Therefore the acceptability criteria are not fulfilled and no conclusion on the genotoxicity of glyphosate can be drawn based on this study.

Appendix to B.6.4 Overview of publications related to genotoxicity that are classified by the applicant as "non-relevant after detailed assessment of full-text article"

To complement the standard toxicity studies, the applicant has performed a literature search in accordance with the EFSA Guidance document EFSA Journal 2011;9(2):2092 "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) 1107/2009". The results were categorized as "non-relevant", as "potentially relevant" or to be of "unclear relevance" following a rapid assessment. For the two latter categories, the full-text documents were reviewed in detail and then categorized as "non-relevant" or "relevant". The articles considered relevant were categorized as A (providing data for establishing or refining risk assessment parameters), as B (articles relevant to the data requirement but in opinion of the applicant only to provide supplementary information that does not alter existing risk assessment) or as C (articles of unclear relevance).

More details on the literature search, including tables describing the studies in categories A, B and C can be found in Volume 3 CA section B.6.10.1.

For one study related to genotoxicity that was classified by the applicant as "non-relevant after detailed assessment of full-text articles", the AGG requested study summaries to further justify the categorization of the information. The justification provided by the applicant was reviewed by the RMS and the assessments are presented in this appendix for this study.

Overview of study categorised by the applicant as non-relevant[13] after rapid assessment of title/abstract for which the RMS requested a summary in order to further justify the categorization.

No.	Technical section	Author	Year	Title
1376	Toxicology and metabolism	Hao Y. et al	2019	Roundup confers cytotoxicity through DNA damage and mitochondria-associated apoptosis induction

Data point	CA 9
Author	Hao Y. *et al.*
Year	2019
Title	Roundup confers cytotoxicity through DNA damage and mitochondria-associated apoptosis induction
Document source	Environmental Pollution (2019), Vol. 252, No. Part A, pp. 917-923
Short description of literature article	In this study, human alveolar carcinoma A549 cells (served as models of alveolar Type II pulmonary epithelium) were selected to detect the Roundup toxicity on lung tissue and its potential risk of inhalation toxicity to humans (e.g. mitochondria associated apoptosis and DNA damage). Alkaline comet assay, immunofluorescence assay and flow cytometric analysis assay were employed as test systems. Roundup (RDP) was obtained from Monsanto (St. Louis, Missouri, USA). Test formulations RDP (0, 50, 75, 100 and 125 mg/mL, calculated based on the active ingredient glyphosate) were freshly prepared in double-distilled water. Trypan Blue, Rhodamine123, phenylmethanesulfonyl fluoride, 4',6-diamidino-2-phenylindole, propidium iodide (PI), and radioimmunoprecipitation assay lysis buffer were obtained from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were obtained from Shanghai Titanchem Co. Ltd. (Shanghai, China). Antibodies were used as follows: caspase-9 (35, 37 and 47 kDa), Bax (20 kDa), Bcl-2 (26 kDa), caspase-3 (35 kDa), cytochrome c (14 kDa), PARP (89 and 116 kDa), gH2AX (14 kDa), and β actin (43 kDa) from Cell Signaling Technology (Beverly, MA, USA). Secondary antibody was obtained from Sangon Biotech

[13] The applicant has provided an Excelsheet in which all articles are presented that are considered non-relevant after rapid assessment (based on title/abstract). These were checked by the RMS and for the articles listed in the table the RMS has requested a summary in order to further justify the categorization of the study.

	Co., Ltd. (Shanghai, China). Human alveolar carcinoma A549 cell line (ATCC, CCL-185) was maintained in DMEM (Hyclone, USA) supplemented with 1% antibiotics (streptomycin and penicillin) (Gibco, USA) and 10% fetal bovine serum (Gibco, USA), and maintained in 37 ºC, 5% CO2 in a humidified atmosphere.
Short description of findings	According to the authors, RDP induced the DNA single-strand breaks and double-strand breaks; the collapse of mitochondrial membrane by increasing Bax/Bcl-2, resulting in the release of cytochrome c into cytosol and then activated caspase-9/-3, cleaved poly (ADP-ribose) polymerase (PARP) in human lung tissue cells. The results demonstrate that RDP can induce A549 cells cytotoxic effects *in vitro* at the concentration lower than the occupational exposures level of workers, which means RDP has a potential threat to human health. However, based on the current research data presented in the study, the authors stated that they could not certain whether the cytotoxic effects of RDP are due to herbicidal active ingredient, adjuvants, or a combination of the two.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication was considered not relevant for the risk assessment and the glyphosate EU renewal because a glyphosate formulation was tested *in vitro* instead of glyphosate. Surfactants present in the formulation induce membrane damage and cytotoxicity, these effects are well established within *in vitro* test systems, and this research is not relevant or informative for the glyphosate EU renewal. In addition, the surfactant system in the formulation tested differs from the EU representative glyphosate formulation MON 52276. 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	The authors of the study aimed to investigate toxicity of a glyphosate formulation on lung tissue and its potential risk of inhalation toxicity to humans. The RMS does agree with the applicant's justification that the study has no relevance to the assessment on glyphosate as the test material was not glyphosate alone or the reference formulation MON 55276. As no individual components were investigated, it is not possible to determine whether any observed effects in this study are related to glyphosate or to one of the other components in the formulation.

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

Refer to separate RAR B.6.5.

B.6.6. REPRODUCTIVE TOXICITY

Refer to separate RAR B.6.6.

B.6.7. NEUROTOXICITY

Refer to separate RAR B.6.7-6.10.

B.6.8. OTHER TOXICOLOGICAL STUDIES

Refer to separate RAR B.6.7-6.10.

B.6.9. MEDICAL DATA AND INFORMATION

Refer to separate RAR B.6.7-6.10

B.6.10. REFERENCES RELIED ON

Refer to separate RAR B.6.7-6.10