Committee for Risk Assessment
RAC

Opinion
proposing harmonised classification and labelling
at EU level of

glyphosate (ISO); *N*-(phosphonomethyl)glycine

EC Number: 213-997-4
CAS Number: 1071-83-6

CLH-O-0000007122-85-01/F

Adopted
30 May 2022

Corrigendum:

The reference to (Brooker et al., 2001) was corrected to (Moxon, 2001) on pages 107 and 110 and details were added to the list of references on page 149.

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **glyphosate (ISO); *N*-(phosphonomethyl)glycine**

EC Number: **213-997-4**

CAS Number: **1071-83-6**

The proposal was submitted by **Sweden** and received by RAC on **15 September 2021**. It was jointly prepared by **France, Hungary, The Netherlands and Sweden**.

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Sweden has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **23 September 2021**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **22 November 2021**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Christine Bjørge**

Co-Rapporteur, appointed by RAC: **Stine Husa** (to 28 February 2022)

  Riitta Leinonen (effective from 1 Mar. 2022)

  supported by **Pietro Paris**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **30 May 2022** by **consensus.**

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	Chemical name	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors and ATE	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	607-315-00-8	glyphosate (ISO); *N*-(phosphonomethyl)glycine	213-997-4	1071-83-6	Eye Dam. 1 Aquatic Chronic 2	H318 H411	GHS09 GHS05 Dgr	H318 H411			
Dossier submitters proposal	607-315-00-8	glyphosate (ISO); *N*-(phosphonomethyl)glycine	213-997-4	1071-83-6	Eye Dam. 1 Aquatic Chronic 2	H318 H411	GHS09 GHS05 Dgr	H318 H411			
RAC opinion	607-315-00-8	glyphosate (ISO); *N*-(phosphonomethyl)glycine	213-997-4	1071-83-6	Eye Dam. 1 Aquatic Chronic 2	H318 H411	GHS09 GHS05 Dgr	H318 H411			
Resulting Annex VI entry if agreed by COM	607-315-00-8	glyphosate (ISO); *N*-(phosphonomethyl)glycine	213-997-4	1071-83-6	Eye Dam. 1 Aquatic Chronic 2	H318 H411	GHS09 GHS05 Dgr	H318 H411			

GROUNDS FOR ADOPTION OF THE OPINION

Process of evaluation

The Combined Draft Renewal Assessment Report and Proposal for Harmonised Classification and Labelling (referred to throughout this opinion as the "CLH dossier") on which this opinion is based was jointly prepared by France, Hungary, The Netherlands and Sweden (further referred to as the Dossier Submitter; DS). This CLH dossier was subject to consultation from 23 September 2021 to 22 November 2021, concurrently through ECHA and EFSA. A further ad hoc consultation[1] was conducted from 29 March to 14 April 2022, on documents potentially relevant to the classification of the substance for the following hazard classes: Respiratory Sensitisation (opened for comments during the ad hoc consultation), Specific Target Organ Toxicity - Single Exposure (respiratory irritation), Germ Cell Mutagenicity, Carcinogenicity, Reproductive Toxicity and Hazardous to the Aquatic Environment.

The draft opinion prepared by the Rapporteurs appointed by RAC was provided to the Committee on 30 March 2022, while a revised section on aquatic environment hazards was provided on 13 May 2022.

The CLH dossier was considered by RAC at its:

- RAC-60 plenary, 16 Mar. 2022 – key issues and stakeholder statements;
- RAC-61 CLH Working Group, 21 and 22 Apr. 2022 – all hazard classes were considered;
- RAC-61 plenary, 30 May 2022 – all hazard classes were considered including the CLH working group's recommendations; the opinion was adopted by consensus.

For reference, the previous RAC opinion on the harmonised classification and labelling (CLH) of glyphosate (ISO); *N*-(phosphonomethyl)glycine (hereafter referred to as glyphosate) was adopted by RAC in 2017 (referred to in this opinion as RAC, 2017), and was based on a CLH dossier submitted by Germany in 2016 (referred to in this opinion as CLH, 2016).

RAC general comments

Toxicokinetics of glyphosate

Absorption

The oral absorption values range between 10.8% and 55% for single dose studies, with a mean of 27% (sexes and different dose levels combined). The oral absorption does not show any sex differences nor a clear difference between the low and the high dose tested. In general, repeated exposure did result in higher absorption values.

Systemic availability

The maximum blood plasma concentration in rats after repeated 14d dietary application of 72 and 385 mg glyphosate/kg bw/d were 0.84 and 5.31 µg/mL for males and 0.64 and 4.69 µg/mL for female rats, respectively (Report no. 00050502, 2020). After a single gavage application of 1 and 100 mg glyphosate/kg bw, maximum plasma concentrations of 0.02 and 8.91 µg/mL for

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[1]

male and 0.036 and 7.63 µg/mL for female rats (Report no. 1413/2-1011, 1996) were determined. After a single gavage application of 10 and 600 mg glyphosate/kg bw, maximum plasma concentrations of 0.22 and 26 µg/mL for male and 0.28 and 29 µg/mL for female rats were determined (CA 5.1.1/010, 1995).

Distribution

The absorbed glyphosate is distributed rapidly; however, only low levels were found in organs and tissues at termination. After a period of 3 - 7 days following oral administration, total body burden accounted for less than 1% of the applied radioactivity. The highest levels were measured in bone followed by kidney, liver and bone marrow after oral or intravenous exposure, see table below from CA 5.1.1/012 (1992). There is no evidence of a potential for accumulation in animals based on residue analysis in organs and tissues after 3 - 7 days. Elimination from bone was shown to be slower than from other tissues. However, the amount of radiolabel in bone after 7 days after a single oral dose was relatively low at 0.02 - 0.03% of the applied dose. The terminal half-lives were comparable (11 and 13h at low and high dose, respectively) when glyphosate was applied via diet for 14 consecutive days (Report no. 00050502, 2020).

Table: *Radioactivity in rat tissue (µg equivalents of [14C]-glyphosate/g) (CA 5.1.1/012, 1992)*

Table 6.1.1.11-28: [^{14}C]-Glyphosate: Absorption, distribution, metabolism and excretion in the rat ▮▮▮▮▮ 1992): Radioactivity in tissues (in µg equivalents of [^{14}C]-glyphosate/g)

Tissue	Intravenous dose* (30 mg/kg bw)		Oral dose* (30 mg/kg bw)		Oral dose** (30 mg/kg bw)		Oral dose* (1000 mg/kg bw)	
	M	F	M	F	M	F	M	F
Blood	0.050	0.084	0.011	0.000	0.000	0.000	0.000	0.000
Bone	4.195	4.355	2.246	2.562	3.096	2.505	56.32	40.66
Bone marrow	0.255	1.264	0.322	0.545	0.325	0.144	3.080	0.000
Brain	0.118	0.120	0.056	0.056	0.019	0.000	0.000	0.000
Abdominal fat	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000
Carcass	0.423	0.335	0.197	0.214	0.339	0.284	5.628	4.476
Heart	0.051	0.025	0.051	0.045	0.000	0.000	0.000	0.000
Kidney	0.304	0.298	0.278	0.205	0.515	0.317	5.170	3.986
Liver	0.241	0.222	0.251	0.254	0.615	0.425	6.144	0.000
Lungs	0.264	0.279	0.124	0.126	0.183	0.173	2.904	1.216
Muscle	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Plasma	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000
Salivary gland	0.082	0.068	0.053	0.079	0.084	0.100	0.000	0.000
Spleen	0.117	0.117	0.140	0.091	0.164	0.153	0.000	0.000
Testes/Ovaries	0.000	0.034	0.000	0.068	0.000	0.028	0.000	0.000
Uterus	N/A	0.248	N/A	0.143	N/A	0.239	N/A	0.000

* Single dose
** Multiple dose (non-radiolabelled glyphosate for 14 consecutive days followed ~24 h later by [^{14}C]-glyphosate)
N/A not applicable

In addition, a study in male and female Sprague-Dawley rats receiving single intraperitoneal injections of radiolabelled ^{14}C-glyphosate was reported by US EPA[1]. Rats were exposed to 1150 mg/kg bw via the intraperitoneal (i.p.) route (no information regarding the number of rats was included). Blood samples were collected 0.25, 0.50, 1, 2, 4, 6 and 10 hours after injection. Femoral bone marrow samples were collected from one third of the male and female rats sacrificed at 0.5, 4, or 10 hours after injection. Thirty minutes after injection of glyphosate, the

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[1] https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/red_PC-417300_1-Sep-93.pdf

concentration of radioactivity in the bone marrow of male and female rats was 0.0044% and 0.0072% of the administered dose, respectively. When assuming first order kinetics, the decrease in radioactivity in the bone marrow occurred with a half-life of 7.6 and 4.2 hours for males and females, respectively. The half-lives of the radioactivity in plasma were approximately 1 hour for both sexes. This study indicates that very low levels of glyphosate reach the bone marrow, and that a rapid elimination from bone marrow occurs (MRID 00132685, 1983).

Metabolism

Very limited metabolism of glyphosate is reported in rats. Most of the glyphosate is eliminated unchanged and a small amount, just under 0.5% of the applied dose, is eliminated as aminomethylphosphonic acid (AMPA). Following 14 days of dietary administration of 72 and 385 mg/kg bw/d glyphosate to rats, no AMPA was detected in plasma at the low dose. At the high dose, AMPA was detected in plasma, and accounted for 0.6% of the systemic exposure to glyphosate (Report no. 00050502, 2020). The maximum blood plasma concentration of AMPA following exposure to 385 mg glyphosate/kg bw/d was about 0.04 µg/mL. The half-life of AMPA was approximately 7 h.

Elimination

Elimination of the unabsorbed fraction of ingested glyphosate via faeces and urine is rapid and nearly complete within 48h. In the urine, 25 - 35% and 53 - 55% is excreted after exposure to 1 and 100 mg/kg bw, respectively. In the faeces, 62 - 73% and 41 - 42% is excreted at 1 and 100 mg/kg bw, respectively. The pulmonary and biliary route of elimination is negligible (Report no. 1413/2-1011, 1996).

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

No classification is proposed for the physical hazards by the dossier submitter (DS). The substance is solid which means that hazard classes related to gases and liquids are not relevant for its physical hazard classification.

Explosives

Glyphosate does not contain chemical groups associated with explosive properties (table A6.1 in Appendix 6 of the UN RTDG). In addition, a GLP study (CSL-21-1120.01, 2021) investigating explosive properties (UN Class 1) of glyphosate, technical substance (wetcake) following guidelines UN Manual of Tests and Criteria, Rev.7 and UN Model Regulations Rev. 21 concluded that the test item does not warrant a classification as explosive according to the CLP Regulation.

Flammable solids

An experimental study (UN test N.1) demonstrated that the pure substance is not flammable (Winkler, 2019).

Self-reactive substances

Glyphosate does not contain chemical groups associated with explosive or self-reactive properties (Tables A6.1 and A6.3 in Appendix 6 of the UN RTDG).

Pyrophoric solids

Experience in manufacture or handling shows that glyphosate does not ignite spontaneously in contact with air at normal temperatures.

Self-heating substances

Based on the negative UN N.4 test glyphosate has no self-heating properties (Winkler, 2019).

Substances which in contact with water emit flammable gases

Knowledge of the substance and experimental studies show the substance does not emit flammable gases when in contact with water. In addition, the chemical structure of glyphosate does not contain metals or metalloids.

Oxidising solids

The negative EEC A.17 result (Wollerton and Husband, 1997) is not sufficient to conclude the substance is not oxidising. The chemical structure contains oxygen atoms which are not bonded only to carbon and hydrogen; therefore, it fails the screening criteria for no classification.

Organic peroxides

Not applicable as the substance does not contain peroxides.

Corrosive to metals

The melting point of glyphosate was determined to be 189.5 °C, which is above the cut-off criteria of 55 °C for testing.

Comments received during consultation

The Glyphosate Renewal Group (GRG)[1] agreed with the DS proposals on all other hazard classes except oxidising solids. They refer to the Renewal Assessment Report (RAR) Vol. I 2.2.1 where it is stated that glyphosate is not an oxidising substance and that results can be used for classification. They also refer to the information in Volume 3 – B.2 where a study performed in accordance with EEC A.17 was accepted for glyphosate. The DS agreed that for the oxidising property the substance cannot be classified as oxidising based on the available test results.

One Member State Competent Authority (MSCA) commented that since glyphosate contains a P-O group the reason for no classification as self-reactive should be changed to "lack to data". The DS explained that the P-O group in glyphosate is a phosphonate functional group (phosphorus in a P^{5+} state) and not a phosphite group (phosphorus in a P^{3+} oxidation state). The phosphite group is known to be of a limited thermal stability. Therefore, the DS considered that a conclusion as 'not classified' is still valid.

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[1] The Glyphosate Renewal Group (GRG) is a collection of companies seeking the renewal of the EU authorisation of the active substance glyphosate in 2022. Current members of the GRG are Albaugh Europe SARL, Barclay Chemicals Manufacturing Ltd., Bayer Agriculture bvba, Ciech Sarzyna S.A., Industrias Afrasa S.A., Nufarm GMBH & Co.KG, Sinon Corporation, Syngenta Crop Protection AG.

Assessment and comparison with the classification criteria

Comparison with the criteria

<u>Explosives</u>

RAC agrees with the DS proposal not to classify glyphosate as explosive. The substance does not fulfil the CLP screening criteria in 2.1.4.3 (a) i.e., due to the absence of chemical groups associated with explosive properties.

<u>Flammable solids</u>

The results of the experimental test do not fulfil the criteria in the CLP Regulation, table 2.7.1. Thus no classification as a flammable solid is warranted.

<u>Self-reactive substances</u>

Instead of the DS proposal "no classification", RAC concludes "no classification due to lack of data" due to the lack of studies in line with the CLP Regulation. According to 2.8.4.2 (a) of the CLP Regulation this classification need not to be applied if there are no chemical groups present in the molecule associated with explosive or self-reactive properties. RAC agrees with the DS response to the consultation comment regarding the phosphonate functional group in glyphosate not being associated with explosive or self-reactive properties. However, uncertainties cannot be ruled out.

<u>Pyrophoric solids</u>

RAC agrees with the DS proposal not to classify glyphosate as a pyrophoric solid. According to the CLP Regulation, 2.10.4.1, this classification does not need be applied when experience in manufacture or handling shows that the substance or mixture does not ignite spontaneously on coming into contact with air at normal temperature.

<u>Self-heating substances</u>

RAC agrees with the DS proposal not to classify glyphosate as a self-heating solid based on the criteria in table 2.11.1 of the CLP Regulation (no positive result in the UN N.4 test).

<u>Substances which in contact with water emit flammable gases</u>

RAC agrees with the DS not to classify glyphosate as a substance that emits flammable gases with water, based on the criteria in the CLP Regulation 2.12.4.1 (a) the chemical structure of the substance or mixture does not contain metals or metalloid and (b) experience in production and handling shows that the substance or mixture does not react with water.

<u>Oxidising solids</u>

RAC agrees with the DS initial proposal in the CLH dossier not to classify glyphosate as an oxidising solid based on lack of data. Glyphosate does not fulfil the criteria in the CLP Regulation 2.14.4.1 and should have been tested according to UN O.1 or UN O.3 method. The negative EEC A.17 test provides supporting information that glyphosate is not oxidising; however, it is not sufficient to conclude on the classification.

<u>Corrosive to metals</u>

RAC agrees with the DS not to classify glyphosate as corrosive to metals. According to the CLP guidance, only solids having a melting point lower than 55 °C (test temperature required in UN Test C.1) must be taken into consideration. No corrosiveness to metals is expected for glyphosate as its melting point is above 55 °C.

Conclusion

Overall, RAC recommends that **no classification for physical hazards of glyphosate is warranted.**

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

The DS summarised 39 acute **oral** toxicity studies in rats and mice of which 27 studies were in accordance with OECD test guidelines and considered as acceptable and four as acceptable with restrictions. All of these studies were included in the RAC evaluation in 2017 (RAC, 2017). The lowest dose resulting in mortality was 2500 mg/kg bw in both mice and rats, but the number of dead animals at this dose was low and many studies demonstrated that most animals tolerated even much higher doses of > 5000 mg/kg bw. Since the LD_{50} values were consistently > 2000 mg/kg bw, the DS concluded that classification for acute oral toxicity was not warranted. The DS noted that clinical signs following oral exposure frequently included diarrhoea, reduced activity, ataxia, piloerection, anogenital staining and hunched posture. In addition, reduced body weight gain was noted in a few studies.

The DS summarised 21 acute toxicity studies in which exposure in rats and rabbits was via the **dermal** route. The only death reported was one female rabbit receiving 5000 mg/kg bw. Fifteen studies in rats were considered as acceptable whereas three studies were acceptable with restrictions. In rabbits the three available studies were considered as acceptable with restrictions. All of these studies were included in the RAC evaluation in 2017 (RAC, 2017). Isolated signs of toxicity comprised body weight loss, diarrhoea, and slight local effects. Since the LD_{50} values were all > 2000 mg/kg bw, the DS concluded that classification for acute dermal toxicity was not warranted.

The DS summarised 17 acute **inhalation** toxicity studies in rats, of which, six studies were considered as acceptable and eleven as acceptable with restrictions. All studies were included in the RAC evaluation in 2017. In eight of them, a concentration > 5 mg/L was tested. The DS therefore considered the information on effects of inhaled glyphosate at high concentrations to be sufficient despite this limit concentration not having been achieved in all experiments. Mortality was confined to three studies (CA 5.2.3/001, 2011; CA5.2.3/012, 1996; CA 5.2.3/021, 1987), but the LC_{50} value in two of the studies was > 5 mg/L and in one study > 1.3 mg/L (the only dose tested); hence, the DS concluded that classification for acute inhalation toxicity was not warranted. Clinical signs included irritation of the upper respiratory tract, hyperactivity, increased or decreased respiratory rate, piloerection, loss of hair, wet fur, slight body weight reduction, slight tremor, and slight ataxia, but the DS noted that these findings were not observed consistently in the studies.

Comments received during consultation

Comments no. 107 - 126 submitted during the consultation were related to the hazard class acute toxicity. Seventeen comments supported the proposal for no classification for acute toxicity via the oral, dermal or inhalation route. These comments were provided by Industry Trade

Organisations, civil society NGOs and individuals. Comment no. 123 from GRG (Company-Manufacturer) also supported the proposed no classification from the DS.

On the request of the DS, the GRG provided the study by Zouaoui *et al.* (2012), which summarises 13 poisoning incidents with glyphosate-based herbicides in France. This publication was evaluated during the previous assessment of glyphosate by the DS (Germany), however, not included in the current assessment by the GRG. Although it is difficult to assign a reliability score to this study, the relevance of the study is limited. The study investigates the symptomatology and blood/urine glyphosate concentrations following oral ingestion of different glyphosate-containing plant protection products, i.e., not the active substance itself. It is not possible to allocate effects to glyphosate, to other components within the formulation, or to a combination of different formulations. The study gives indications that glyphosate is converted only to a very limited extend to AMPA in humans; however the results need to be considered with caution. Nevertheless, it is noted that the results are in line with the available data from rats and with the *in vitro* comparative metabolism study, which also indicated limited metabolism of glyphosate in mammals (Report no. S19-04081).

In addition, on the request from the DS, the GRG provided clarification regarding the use of the same batch of test item, since different conclusions were drawn regarding the purity and the acceptability of acute toxicity studies. The GRG explained that the study summaries incorrectly stated that they should be considered "supportive due to low purity." Instead, they should indeed be considered acceptable. This applies to studies CA 5.2.1/020, 1994; CA 5.2.3/016, 1994; CA 5.2.4/012,1994; CA 5.2.5/015, 1994; CA 5.2.6/016, 1994.

Assessment and comparison with the classification criteria

RAC noted that no new acute toxicity studies were submitted compared to the previous RAC evaluation (see RAC, 2017)

Human data

In the CLH dossier, no studies or case reports were found where humans were exposed to glyphosate itself at single doses. However, a number of poisoning incidents have been reported following accidental or intentional intake of formulated glyphosate-based herbicides, mostly via the oral route but also some by inhalation. Importantly, the doses in these poisoning incidents were not reported. Furthermore, it is not possible to clearly distinguish between effects due to exposure to glyphosate and those related to exposure to co-formulants.

Animal data

The DS has included several acute toxicity studies, mostly in rats following oral, dermal and inhalation exposure. In addition, studies in mice following oral exposure, and in rabbits following dermal exposure were included.

Oral exposure

For the assessment of acute toxicity following oral exposure to glyphosate, 23 studies in rats and four in mice were included by the DS and considered to be acceptable, and four studies in rats and one study in mice acceptable with restriction (table 18, CLH dossier). Twelve of the acute toxicity tests in rats were performed with only one concentration (limit test or fixed dose test) with LD_{50} values > 2000 mg/kg bw and 12 with an LD_{50} value of > 5000 mg/kg bw. In the remaining acute toxicity tests the LD_{50} values ranged from > 5000 to > 8000 mg/kg bw. Two acute oral toxicity studies were performed in mice as limit tests with LD_{50} values > 2000 mg/kg bw and two studies with a limit test with LD_{50} values > 5000 mg/kg bw. In the fourth acute toxicity test in mice, an LD_{50} value > 7500 mg/kg bw was set with mortality, lethargy, ataxia, dyspnoea and weight loss observed at ≥ 2500 mg/kg bw.

The most frequent toxic signs reported in the acute toxicity tests with oral exposure were, diarrhoea, reduced activity, ataxia, piloerection, convulsions, and hunched posture. Mortality was reported in one study in rats with mortality in 1/10, 1/10, 3/1, 7/10 and 10/10 animals at 2500, 3500, 5000, 7000 and 9000 mg/kg bw respectively. In mice, mortality was also reported in one study ≥ 2500 mg/kg bw.

RAC concludes that following oral exposure to glyphosate, LD_{50} values in rats and mice were consistently above 2000 mg/kg bw which, according to the CLP Regulation, is the upper threshold for classification for acute toxicity following oral exposure. Therefore, RAC agrees with the DS that **no classification for acute toxicity via the oral route is warranted**.

Dermal exposure

For the assessment of acute toxicity following dermal exposure to glyphosate, 18 studies in rats and three in rabbits were included by the DS (table 21, CLH dossier). Sixteen of the studies in rats were performed with one high dose of glyphosate (limit test) with LD_{50} values > 2000, > 5000 or > 5050 mg/kg bw. In two studies with several doses of glyphosate the LD_{50} values were > 5000 and 8000 mg/kg bw. No mortality related to glyphosate exposure was reported in the studies. In rabbits, the LD_{50} value was > 5000 mg/kg bw, with mortality at day 13 in one female rabbit in study CA 5.2.2/023 (1988), and at day three in study CA 5.2.2/025 (1987) at 5000 mg/kg bw which was considered not related to glyphosate exposure since no internal abnormalities were noted during gross examination.

The most frequent toxic signs reported in the acute toxicity tests with dermal exposure were body weight loss, diarrhoea, anorexia and slight local effects. RAC concludes that following dermal exposure to glyphosate, LD_{50} values in rats and rabbits were consistently above 2000 mg/kg bw which, according to the CLP Regulation, is the upper threshold for classification for acute toxicity following dermal exposure. Therefore, RAC agrees with the DS that **no classification for acute toxicity via the dermal route is warranted**.

Inhalation exposure

For the assessment of acute toxicity following inhalation exposure to glyphosate, 17 studies in rats were included by the DS (table 24, CLH dossier). In eight of the studies only one concentration at approximately 5.0 mg glyphosate/L was tested and all LC_{50} values were ≥ 5.0 mg/L. Of the remaining studies, three studies were performed with a concentration of glyphosate of approximately 2.0 mg/L with LC_{50} values > 2.0 mg/L and one study with an LC_{50} value of > 3.25 mg/L. One study had two concentrations of glyphosate with LC_{50} values > 4.43 mg/L, the highest concentration tested. Two studies had one single concentration and reported LC_{50} concentrations of > 1.9 mg/L and 1.3 mg/L. RAC notes that there were nine studies in which a median aerodynamic diameter (MMAD) < 4 μm was employed and which were performed according to the OECD TG 403 requirements. A greater weight was put on these studies by RAC. There were six studies in which an MMAD > 4 μm was used and two studies without any information regarding the MMAD. RAC also noted that data from sprayed aerosols were lacking in the CLH dossier.

The most frequent toxicological signs reported in the acute toxicity tests with inhalation exposure were irritation of the upper respiratory tract, hyperactivity, increased or decreased respiratory rate, piloerection, loss of hair, wet fur, slight body weight reduction, slight tremor and slight ataxia. The clinical signs were not reported consistently among the studies. Mortality was reported in three studies; in the first study, 2/5 males and 2/5 females died at 4.43 mg/L; in the second study, only 1/5 female died at 5.04 mg/L. The incidence of deaths in the two studies did not result in LC_{50} values below 5.0 mg/L, and in the third study 1/5 female died at 1.3 mg/L. The two first studies used glyphosate from the same source and used nose-only exposure. The last study used a Rodeo Herbicide with isopropylamine salt of glyphosate, 42.2% and whole-body exposure.

RAC concludes that following inhalation exposure to glyphosate no LC$_{50}$ values in rats were reported to be below 5.0 mg/L which, according to the CLP Regulation, is the upper threshold for classification for acute toxicity (dust and mists) following inhalation exposure. Therefore, RAC agrees with the DS that **no classification for acute toxicity via the inhalation route is warranted**.

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

Based on a large number of acute toxicity studies in rats, mice and rabbits, in which non-lethal effects were confined to very high doses and were non-specific, the DS concluded that classification for STOT SE (categories 1 or 2) was not appropriate. In support of this argument, no evidence of neurotoxicity was observed in an acute neurotoxicity study in rats at doses up to 2000 mg/kg bw (CA 7.7.1/001).

The DS also concluded that no classification for respiratory tract irritation was warranted (STOT SE 3; H335), since there was no evidence for respiratory tract irritation by the active substance in humans but acknowledged that "such an exposure will seldom occur". The DS suggested that reported cases of possible respiratory tract irritation were from formulations containing polyoxyethylenealkylamine (POEA) surfactants. There was, however, no data to confirm if this was indeed the case.

The DS further noted that there was no evidence of narcotic effects observed in any of the evaluated studies (STOT SE 3; H336).

Comments received during consultation

Comments no. 188 - 208 submitted during the consultation were related to the hazard class STOT SE. Seventeen comments supported the proposal for no classification for STOT SE. These comments were provided by Industry Trade Organisations, civil society NGOs and individuals. Comment no. 204 from the GRG (Company-Manufacturer) also supported the proposed no classification from the DS.

Comment no. 206 from an Academic institution, although providing no view as regards the classification for STOT SE, indicated that there is a weak presumption of a link between glyphosate exposure and an excess risk of wheezing (allergic or not) and asthma. It was noted, however, that this conclusion is based on the results of a limited number of epidemiological studies, most of which are from the Agriculture Health Study cohort (AHS). Furthermore, they stated that experimental toxicology studies show that glyphosate has pro-oxidant and mitotoxic effects, which could be involved in pathophysiology of asthma and chronic obstructive pulmonary disease.

Assessment and comparison with the classification criteria

Several acute toxicity studies in rats, mice and rabbits were briefly described by the DS to illustrate transient, non-lethal and unspecific effects (associated with high doses of glyphosate) that were not sufficient for classification as STOT SE 1 or 2. Supporting evidence for no classification was also found in an acute neurotoxicity study in rats where no neurotoxicity was reported at dose levels of 500, 1000 or 2000 mg/kg bw. Furthermore, no clinical signs were

reported after the first exposure from many repeated dose toxicity studies where lower doses were applied.

As regards classification with STOT SE 3 (narcotic effects), no narcotic effects were reported in any of the toxicity studies.

Further consideration was given to a classification with STOT SE 3 for respiratory tract irritation. Clinical signs were reported in a variety of acute inhalation studies performed on rats. Vague and general effects on breathing were described as clinical signs in seven out of 17 inhalation toxicity studies according to the CLH dossier. These effects were not consistent. The studies were all performed with glyphosate and were all guideline- (and GLP-) compliant. In three studies (CA 5.2.3/001, 2011; CA5.2.3/012, 1996; CA 5.2.3/021, 1987) mortalities were observed and the clinical signs were more pronounced.

Pathology findings (dark lungs) were reported in two studies (CA 5.2.3/012, 1996; CA 5.2.3/016, 1994). In the first study, dark lungs were reported in the two male rats that died. In the other study, dark area (1/5 male, 1/5 female rats) or multiple dark foci in the lungs (0/5 male and 4/5 female rats) were reported. The remaining 14 acute inhalation toxicity studies showed no pathological findings.

There was no evidence of respiratory tract irritation in humans following exposure to glyphosate. In the CLH dossiers from 2016 and 2021, the DS included one case of respiratory tract irritation (Burger *et al.*, 2009), which was evaluated by RAC in 2017. The respiratory tract irritation was considered to be due to exposure to a formulated mixture and not solely the active substance glyphosate. The authors speculated that the effect was due to polyethoxylated alkylamine (POEA) non-ionic surfactants. In any case, this particular study did not provide any significant information to compare with the classification criteria.

In summary, there were no clear human data to support classification for respiratory tract irritation. There were no specific data which clearly indicated respiratory tract irritation in studies with animals. A variety of clinical signs were observed across a number of acute studies (slight dyspnoea, decreased or increased respiratory rate, breathing effects, irregular breathing, rales, laboured respiration, gasping respiration), but they were not always consistent and did not always occur together but in isolated studies. There is a general lack of pathology examinations in the studies (lung pathology was recorded in only two out of 17 studies) and it is difficult to rule out the possibility that isolated idiosyncratic reactions or responses triggered in hypersensitive test subjects were being observed. All effects appear to have been transient in nature. In conclusion, there is not sufficient evidence amongst these studies to meet the CLP criteria for classification.

No new studies were included compared to 2016 CLH dossier (CLH, 2016). RAC concludes that **classification for specific target organ toxicity - single exposure is not warranted**, based on the results from the acute and the repeated dose toxicity studies when compared with the CLP criteria.

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

The DS reported that out of 23 studies addressing skin irritating effects of glyphosate, 18 were assessed to be in accordance with OECD test guidelines and to be acceptable. All studies consistently showed no or only very slight skin irritation potential with the highest mean irritation score of 0.33 for erythema which is below the criteria for classification. No human data on skin

effects after exposure to non-formulated glyphosate alone were reported. Therefore, no classification was proposed for skin corrosion/irritation.

Comments received during consultation

Comments no. 127 - 146 submitted during the consultation were related to the hazard class skin corrosion/irritation. Seventeen comments supported the proposal for no classification for skin corrosion/irritation. These comments were provided by Industry Trade Organisations, civil society NGOs and individuals.

Comment no. 143 from the GRG (Company-Manufacturer) also supported the proposed no classification from the DS.

Assessment and comparison with the classification criteria

Eighteen guideline-compliant studies with rabbits were summarised by the DS (table 27, CLH dossier). From these, 15 studies were negative. Two studies (CA 5.2.4/009, 2005; CA 5.2.4/001, 2011), both consistent with OECD TG 404, and a third one (CA 5.2.4/012, 1994) according to US EPA Guideline, each showed very slight erythema. In CA 5.2.4/009, one animal showed very slight erythema (score 1) one hour after patch removal which was cleared from this animal by 24 hours. No signs of irritation were observed in the other treated animals. In CA 5.2.4/001 (2011), very slight erythema (score 1) was observed in one animal at 1 and 24 hours after patch removal. No signs of irritation were observed in the other treated animals. In the third study (CA 5.2.4/012, 1994) very slight erythema was noted in two animals one hour after patch removal, which persisted at the 24h observation in one animal and was also noted in one other animal. No other skin reactions were noted for any animals at any other observation time point. Classification is triggered where a mean value of $\geq 2.3 - \leq 4.0$ for erythema/eschar or for oedema in at least two of three tested animals from gradings at 24, 48 and 72 hours is observed, and hence, the results do not meet the criteria for classification for skin irritation Category 2.

There is no information on skin corrosion/irritation in humans exposed to non-formulated glyphosate alone.

No additional studies were included compared to the RAC evaluation in 2017. In conclusion, RAC agrees with the DS that **no classification for skin corrosion/irritation is warranted**.

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

Glyphosate has an existing harmonised classification for Eye Damage 1. The DS included 26 eye irritation studies, of which 18 were assessed to be acceptable or acceptable but with restrictions. Within the acceptable studies, eye irritation or serious eye damage was observed in 14 studies; one study revealed corrosive properties, but the three remaining studies were negative for eye irritation. As regards the unacceptable studies, three were negative, one was positive for eye irritation, and two were positive for eye damage. The DS noted, however, that in these studies, rinsing of the eyes was performed one hour after instillation, while according to OECD TG 405 the eyes should be rinsed after 24 hours. On the other hand, in many studies, there was no rinsing at all. The DS therefore assumed that the different outcomes could be explained by methodological differences. Two further studies were negative; however, the purity of the test substance in these studies were low, and these studies were considered to be of low relevance.

The DS noted that the majority of studies showed a potential for serious eye damage or eye irritation and the criteria for Eye Damage 1 were met in six studies, whereas the results from eight positive studies could instead support classifying glyphosate in Category 2 (Eye Irritation). Further, the DS noted that no human cases of eye effects after exposure to non-formulated glyphosate were reported and that no human data relevant for classification were available.

The DS concluded that since evidence of strong eye irritation was obtained in several (albeit not in all) studies, classification for Eye damage in Category 1 was warranted.

Comments received during consultation

Comments no. 147 - 167 submitted during the consultation were related to the hazard class serious eye damage/irritation. Eighteen comments supported the DS proposal for classification for Eye Damage 1. These comments were provided by Industry Trade Organisations, civil society NGOs and individuals.

Comment no. 164 from the GRG (Company-Manufacturer) also supported the proposed classification from the DS, however, with the caveat that this conclusion applies for glyphosate (which is an acid) but not for its salts. They were of the opinion that the glyphosate salts should not be classified for serious eye damage/irritation since they are the neutralised form of glyphosate (acid) with completely different properties.

Assessment and comparison with the classification criteria

Glyphosate was classified in 1999 by the Technical Committee for Classification and Labelling (TC C&L) of the European Chemicals Bureau with Xi; R41 (Risk of serious damage to eyes). According to the CLP Regulation, this classification corresponds to Eye Damage 1; H318 (Causes serious eye damage). In the RAC opinion from 2017, the classification as Eye Damage 1; H318 (Causes serious eye damage) was confirmed. In their current evaluation, the DS included 26 eye irritation studies, 18 of which were assessed to be acceptable or acceptable with restrictions.

Table: *Summary of animal studies on serious eye damage/eye irritation (revised from table 30 in the CLH dossier).*

Study	Strain, number of animals	Purity	Amount applied	Effects / Result
CA 5.2.5/001, 2011 (Study considered acceptable by DS with the note that due to low pH of 1.99 the study should not have been conducted)	NZW rabbit 1 male	96.3%	Undiluted solid glyphosate 100 mg No rinsing of eyes	Based on results in one animal, the study was terminated at 24h: corneal opacity & erosion (3); conjunctiva: redness (3), chemosis (4), discharge (3), few black points; oedema of the eyelids; positive fluorescein staining at 24 h. Fulfils the criteria for Category 1
CA 5.2.5/002, 2010 (Study considered acceptable by DS)	Himalayan rabbit 3 males	97.3%	Undiluted solid glyphosate 100 mg Eyes washed with NaCl 1h post dosing	The mean scores were as follow: cornea opacity (1.00, 1.00, 1.00), iris lesions (0.67, 0.67, 1.0), conjunctivae redness (1.00, 1.33, 2.00) and chemosis (0.33, 0.33, 0.33). These effects were fully reversible within 7 days. Fulfils the criteria for Category 2
CA 5.2.5/003, 2009 (Study considered acceptable by DS)	Himalayan rabbit 3 males	96.4%	Undiluted solid glyphosate 100 mg	Slight signs of ocular changes, reversible within 8 days. The individual mean scores over 24, 48 and 72h were as follow: corneal opacity (1.00, 1.00, 1.00), conjunctival redness

Study	Strain, number of animals	Purity	Amount applied	Effects / Result
			Eyes rinsed 1h post dosing	(1.00, 1.00, 1.00), iris lesions (0.33, 0.67, 1.00), and chemosis (0.00, 0.33, 0.67). Fulfils the criteria for Category 2
CA 5.2.5/004, 2009 (Study considered acceptable by DS)	Himalayan rabbit 3 males	98.8%	Undiluted solid glyphosate 100 mg Eyes rinsed 1h post application	The individual mean scores over 24, 48 and 72h for the three animals were as follow: corneal opacity corneal opacity (1.00, 0.00, 0.67), iris lesions (0.00, 0.00, 0.00), conjunctival redness (1.00, 0.67, 0.67), and conjunctival chemosis (0.67, 0.00, 0.00). Results do not meet classification criteria
CA 5.2.5/005, 2009 (Study considered acceptable by DS)		96.66%	Glyphosate technical	pH measurement was performed with the test item in a 1% (w/w) solution in purified water before the study initiation. The pH of the test item was found to be 1.93. No test performed due to the low pH.
CA 5.2.5/006, 2009 (Study considered acceptable by DS)	NZW rabbit 2 males, 1 female	96.4%	Glyphosate, tech grade mixed 5-batch 0.1 mL (93.2 mg)	The individual mean irritation scores (24, 48 and 72h) of the three rabbits were corneal opacity (1.00, 0.00, 2.00), iris lesions (0.00, 0.00, 1.00), conjunctival redness (2.00, 0.67, 2.00), and chemosis (1.67, 0.00, 3.00). The effects were reversible within 17 days. Fulfils the criteria for Category 2
CA 5.2.5/007, 2008 (Study considered acceptable by DS)	NZW rabbit 1 male, 1 female	98.05%	Undiluted solid glyphosate technical 100 mg	Only 2 animals due to severe effects: The individual mean scores over 24, 48 and 72h for each animal were corneal opacity (3.33, 3.67), iris lesions (1.00, 1.00), conjunctiva redness (3.00, 2. 67), and chemosis (1.33, 2.00). These effects were not reversible within 21 days. Fulfils the criteria for Category 1
CA 5.2.5/008, 2007 (Study considered acceptable by DS)	NZW rabbit 2 males, 1 female	96.1%	Undiluted solid glyphosate, technical 100 mg	Mild, early onset and transient ocular changes (reversible within 7 days). Corneal opacity (0.00), iritis lesions (0.00), conjunctiva redness (1.34), chemosis (0.44). Results do not meet classification criteria
CA 5.2.5/009, 2007 (Study considered acceptable by DS)	NZW rabbit 1 male, 2 females	95.1%	Undiluted solid glyphosate, technical 100 mg	Marked, early onset and transient ocular changes. Mean scores were as follow: cornea opacity (0.67, 1.67, 2.00), conjunctival redness (2.00, 2.00, 2.67), chemosis (2.00, 2.00, 1.00), reversible within 10 days, no signs of corrosion or staining. Fulfils the criteria for Category 2
CA 5.2.5/010, 2005 (Study considered acceptable with restrictions by DS)	NZW rabbit 3 males	97.23%	Powdered glyphosate acid technical 0.1 mL (60 mg)	All animals: corneal opacity, iris lesions, conjunctival redness & chemosis, reversible within 10 days. The individual mean scores over 24, 48 and 72h for each animal were: corneal opacity (1.00, 1.00, 1.00), iris lesions (1.00, 1.00, 1.00), conjunctiva redness

Study	Strain, number of animals	Purity	Amount applied	Effects / Result
				(2.33, 2.67, 2.67), and chemosis (1.67, 2.00, 2.00). These effects were fully reversible within 10 days. Fulfils the criteria for Category 2
CA 5.2.5/011, 1997 (Study considered acceptable by DS)	NZW rabbit 6 females	95.6%	Undiluted solid glyphosate acid 100 mg	Mean scores were; corneal opacity (1.3), iritis 0.7), conjunctival redness (1.9) and chemosis (1.4). All effects reversible within 8 days. Fulfils the criteria for Category 2
CA 5.2.5/012, 1996 (Study considered acceptable by DS)	NZW rabbit 6 males, 3 females	98.2%	0.1 mL (Equivalent to 65 mg undiluted solid glyphosate)	Severely irritant in unwashed eyes: corneal opacity, conjunctival redness, chemosis, not reversible within 21 days (2 females); moderate irritation in washed eyes (washed after 30s), reversible within 21 days. The individual mean scores at 24, 48 and 72h for the animals were corneal opacity (1.00, 1.00, 2.00, 1.00, 1.00, 1.00), iris lesions (0.00 for all animals), conjunctival redness (2.67, 3.00, 2.67, 3.00, 2.33, 3.00), and chemosis (1.67, 2.67, 2.33, 2.00, 2.00, 2.33). Fulfils the criteria for Category 1
CA 5.2.5/013, 1995 (Study considered acceptable by DS)	NZW rabbit, 12 females	97.56%	Undiluted solid glyphosate technical 100 mg (pure)	Six females without eye irritation. Mean scores were: cornea opacity (2.00, 2.67, 2.00, 2.00, 2.00 1.67, not reversible within 21 days (3/6 females)), iris lesions (1.00 (in 5 females), 0.67 (in one female), reversible within 10 days), conjunctival redness (2.00 in all females and reversible within 16 days), conjunctival chemosis (2.00, 1.67, 2.33, 2.33, 2.00, 1.67, reversible within 7 days). Six females with eye irrigation (30 s & 2 min. post application): reduced effects and faster recovery. Fulfils the criteria for Category 1
CA 5.2.5/014, 1994 (Study considered supplementary by DS)	NZW rabbit 3 males, 3 females	62.2% as glyphosate isoproylamine salt and 46.1% as glyphosate	Undiluted glyphosate premix (technical concentrate) 0.1 mL	The individual mean scores at 24, 48 and 72h for the animals were 0.00 for corneal opacity, iris lesions, conjunctival redness and conjunctival chemosis. Results do not meet classification criteria
CA 5.2.5/016, 1994 (Study considered acceptable with restrictions by DS)	NZW rabbit 4 females	99.6%	Solid undiluted glyphosate, technical 0.1 g	The individual mean scores at 24, 48 and 72h for the animals were corneal opacity (2.00, 1.00, 1.33, 1.00), iris lesions (1.00, 1.00, 0.33, 1.00), conjunctival redness (1.00, 1.67, 2.00, 2.00), and chemosis (2.00, 1.67, 2.00, 3.00). Fulfils the criteria for Category 2
CA 5.2.5/019, 1990 (Study considered acceptable with restrictions by DS)	NZW rabbit 3 females	98.1%	Undiluted solid glyphosate, technical 0.1 g	The individual mean scores at 24, 48 and 72h for the animals were corneal opacity (1.00, 1.00, 1.67), iris lesions (0.00, 0.00, 0.67), conjunctival redness (1.00, 1.00, 1.33), and chemosis (0.67, 0.67, 1.00).

Study	Strain, number of animals	Purity	Amount applied	Effects / Result
				All signs of irritation were cleared by day 8. Fulfils the criteria for Category 2
CA 5.2.5/020, 1989 (Study considered acceptable with restrictions by DS)	NZW rabbit 1 male	98.6%	Undiluted solid glyphosate, technical 0.1 g	Study terminated after 4 days due to the degree of eye irritation observed. The individual mean scores at 24 and 72h for the animals were 1.00 for corneal opacity and iris lesions, and 2.00 for conjunctival redness and conjunctival chemosis. Corneal opacity persisted until termination of the study (day 4). Fulfils the criteria for Category 1
CA 5.2.5/022, 1988 (Study considered acceptable with restrictions by DS)	NZW rabbit 6 animals, sex distribution unknown	97.76%	Undiluted solid glyphosate 100 mg	One rabbit died: considered not treatment related. The individual mean scores over 24, 48 and 72h for each animal were: corneal opacity (2.67, 1.67, 2.00, 1.00, 2.33, 2.67), iris lesions (0.00, 0.00, 1.00, 0.00, 0.00, 0.00), conjunctival redness (2.0 for all animals), and chemosis (2.00, 3.33, 3.33, 2.67, 2.00, 2.00). Some effects were not reversible within 21 days. Fulfils the criteria for Category 1
CA 5.2.5/023, 1987 (Study considered supplementary by DS)	NZW rabbit 6 animals, sex unknown	70.7%	Undiluted solid glyphosate 0.1 g	The individual mean scores at 24, 48 and 72h for all animals were 0.00 for corneal opacity, iris lesions, and chemosis and conjunctival redness (0.33, 0.00, 0.00, 0.00, 0.00, 0.00). Results do not meet classification criteria
CA 5.2.5/024, 1987 (Study considered acceptable with restrictions by DS)	NZW rabbit 6 animals, sex unknown	90.8%	Undiluted solid glyphosate 0.1 g	The individual mean scores at 24, 48 and 72h for the animals were 0.00 for corneal opacity, iris lesions and chemosis, and for conjunctival redness (0.33, 0.67, 0.33, 0.33, 0.67, 0.33). Results do not meet classification criteria

As regards studies assessed to be acceptable, four were negative for eye irritation while 14 studies were unequivocally positive. In addition, one study considered supplementary was negative and six studies were considered unacceptable. The severity of eye damage and reversibility of effects determines whether classification in Category 1 or 2 is most appropriate.

The criteria for Category 1 and 2 are described in Annex 1 of the CLP Regulation, tables 3.3.1 and 3.3.2, respectively.

One study (CA 5.2.5/005, 2009) showed that glyphosate is corrosive based on a pH of 1.93. Six studies (CA 5.2.5/001, 2011; CA 5.2.5/007, 2008; CA 5.2.5/012, 1996; CA 5.2.5/013, 1995; CA 5.2.5/020, 1989; CA 5.2.5/022, 1988) showed a range of severe effects in the rabbits' eyes including corneal opacity, iritis, conjunctival hyperaemia, chemosis and secretion that were not reversed after 21 days and scores which meet the criteria for classification in Category 1.

Further, eight studies (CA 5.2.5/002, 2010; CA 5.2.5/003, 2009; CA 5.2.5/006, 2009; CA 5.2.5/009, 2007; CA 5.2.5/010, 2005; CA 5.2.5/011, 1997; CA 5.2.5/016, 1994 and CA

5.2.5/019, 1990) showed irritation scores in support of classification in Category 2. For the rest of the studies, no Category can be assigned due to limited reporting of the data.

In summary, six studies fulfilled the CLP criteria for classification in Category 1, while another group of eight studies fulfilled the criteria for Category 2 and a third group of three studies were negative. No clear correlation was observed between classification outcome and rinsing since studies with early rinsing (ranging from 30 seconds to 1 hour) and studies with rinsing at 24 hours or no reported rinsing met the criteria for either Category 2 classification or no classification.

No human cases of eye effects after exposure to non-formulated glyphosate alone were reported, and no human data relevant for classification are available. RAC noted that no additional studies were included compared to the RAC evaluation in 2017.

In conclusion, a number of studies of acceptable quality provided clear evidence that glyphosate meets the criteria for classification as Eye Dam. 1. Overall, the results from the studies assessed for eye irritation/eye damage by RAC did not contradict the existing classification of glyphosate in CLP Annex VI, and RAC agrees with the DS that **classification for eye damage 1 (H318; causes serious eye damage) is warranted** and the current classification should be retained.

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

The DS noted that an appropriate animal model for respiratory sensitisation is not available and that there is no evidence of respiratory sensitisation in humans arising from exposure to glyphosate.

Comments received during consultation

Comment no. 206 from an Academic institution indicates that there is a weak presumption of a link between glyphosate exposure and an excess risk of wheezing (allergic or not) and asthma. It was noted, however, that this conclusion is based on the results of a limited number of epidemiological studies, most of which are from the AHS cohort. Furter, they stated that experimental toxicology studies shows that glyphosate has pro-oxidant and mitotoxic effects, which could be involved in pathophysiology of asthma and chronic obstructive pulmonary disease. No position as regards the classification for respiratory sensitisation was indicated in this comment.

Assessment and comparison with the classification criteria

There are no data available which indicate that glyphosate causes respiratory sensitisation. No classification proposal was presented for this hazard class and no data were provided in the CLH dossier. For the classification for STOT RE, a study in mice was included studying the mechanism of airway inflammation following intranasal exposure to 0, 0.1, 1 or 100 µg glyphosate in female mice (Kumar *et al.*, 2014). Since this study is related to airway inflammation, the study is also included for this hazard class, see below.

RAC notes that during the consultation one Academic institution raised the issue of respiratory sensitisation with the presumption of a weak link between glyphosate and respiratory health. A limited number of epidemiological studies, including the AHS cohort, show a weak increased risk of wheeze (allergic or non-allergic) and asthma.

Kumar *et al.* (2014) studied the mechanism of airway inflammation following intranasal exposure to 0, 0.1, 1 or 100 µg glyphosate in female mice (8 mice/group): C57BL/6 wild type (WT) and TLR4-/- mice and BALB/c female WT mice and IL-13-/- mice were exposed daily for 7 days or 3 times/week for 3 weeks. The study was considered as acceptable with restrictions by the DS . The cellular response, humoral response and lung function of the mice were assessed. Exposure to 1 or 100 µg glyphosate resulted in increased total cell count/lung, as well as eosinophil and neutrophil counts compared to controls in WT mice, however, without a clear dose-response. No changes in the number of mast cells were reported. Further, exposure to glyphosate induced pulmonary IL-13-dependent inflammation and promoted Th2 type cytokines, but not IL-4. No effect was seen at 0.1 µg. IL-33 and TSLP (involved in airway inflammation) were increased in the respiratory epithelium of glyphosate-treated wild-type mice, and inflammation was confirmed by histological examination. The study concluded that exposure to glyphosate induced minor exacerbation of immune response in WT female mice. RAC notes that glyphosate was administered (30 µL) to the nose of anesthetised mice in order to aspirate the solution, and it is unclear how aspiration of glyphosate is related to an exposure to glyphosate via inhalation.

Hoppin *et al.* (2017) investigated the association of pesticide use and allergic and non-allergic wheeze among male farmers in a prospective study using interview data from 2005 - 2010 from the AHS cohort. 22134 male farmers were included in the study with approximately 60% current users of glyphosate-based herbicides. Glyphosate was found to be associated with increased risk of allergic as well as non-allergic wheeze (odds ratio (OR)=1.56; 95% confidence interval (CI) 1.19 - 2.03 and OR=1.24, 95% CI: 1.07 - 1.44, respectively with a *p*-value of 0.12). It is noted that the exposure is related to a glyphosate formulation and not glyphosate as such. The models were adjusted for body mass index (BMI), current asthma, age, smoking status, state (North Carolina or Iowa), days applied pesticides and days they drove diesel tractors.

Hoppin *et al.* (2006) identified, when using a two-pesticide model, that chlorimuron-ethyl was a confounder of all of the herbicide associations including glyphosate. The study did not find any association between glyphosate exposure and wheeze when controlling for exposure to chlorimuron-ethyl in addition to age, smoking status, asthma/allergy and BMI in 2255 commercial pesticide applicators.

Hoppin *et al.* (2008) however found an association between exposure to glyphosate-based herbicide and atopic asthma (OR=1.31, 95% CI: 1.02 - 1.67) when investigating 25814 farm women and adjusting for age, state, smoking status, BMI and growing up on a farm.

Further, Henneberger *et al.* (2014) observed a decreased risk of asthma exacerbation among 926 AHS adult pesticide applicators with active asthma exposed to glyphosate-based herbicide (OR=0.5, 95% CI: 0.3 - 0.8).

Patel *et al.* (2018) found a positive association, however not statistically significant, between glyphosate use and increased risk of current asthma (OR=1.3, 95% CI: 0.97 - 1.8). The model was adjusted for sex and region. A population of 11210 farm operators were included in the study.

RAC is of the opinion the study assessing mechanisms of airway inflammation in mice following internasal exposure to glyphosate is not relevant for classification for respiratory sensitisation. Furthermore, the studies mainly come from the AHS cohort, showing only a weak correlation between exposure to glyphosate-based herbicide (a glyphosate containing formulation) and allergic and non-allergic wheeze or atopic asthma and RAC considers that this is not sufficient for a classification for respiratory sensitisation. It is further noted that no information on respiratory sensitisation is available on glyphosate as such. In conclusion, RAC agrees with the DS that **no classification for respiratory sensitisation is warranted based on insufficient data**.

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The 16 studies which were considered to be acceptable or acceptable with restrictions, including the Magnusson & Kligman Guinea Pig Maximisation Tests (GPMT) and Local Lymph Node Assays (LLNA), addressing the skin sensitisation potential of glyphosate, were all negative. However, the DS noted that one Buehler test (CA 5.2.6/011, 2005) was equivocal. In addition, six studies which were considered to be supplementary or not acceptable included one additional Buhler test (CA5.2.6/018, 1992) which showed equivocal results.

Overall, based on the large majority of negative studies the DS did not propose classification for skin sensitisation.

Comments received during consultation

Comments no. 168 - 187 submitted during consultation were related to the hazard class skin sensitisation. Eighteen comments supported the DS proposal for no classification for skin sensitisation. These comments were provided by Industry Trade Organisations, civil society NGOs and individuals. Comment no. 184 from the GRG (Company-Manufacturer) also supported the proposed no classification from the DS.

Assessment and comparison with the classification criteria

Two LLNA studies and 13 GPMT studies were included by the DS for the assessment of skin sensitisation (table 36, CLH dossier). All studies were negative. In the GPMT studies the intradermal induction doses ranged from 0.01% to 25% and the vehicle was either arachis oil, propylene glycol, water, PEG-300, paraffin oil, white petrolatum, or isotonic saline. The challenge doses ranged from 15% to 75% glyphosate. In the LLNA studies, the glyphosate acid dose levels used were 0, 10, 25, 45 or 50 (%w/v). Hexylcinnamaldehyde was included as a positive control and demonstrated skin sensitisation.

The DS reported that two Buehler tests (CA 5.2.6/011, 2005; CA 5.2.6/018, 1992) performed with glyphosate were equivocal. One of the studies was considered acceptable and the other unacceptable by the DS. In the RAC assessment in 2017, information regarding these Buhler tests was not included in the CLH dossier because the results from the LLNA and GPMT studies were considered to be more rigorous than those from a Buhler test. A faint skin reaction was observed in 6/20 and 4/10 animals, respectively, which were scored as 0.5 in all animals. It is noted that according to OECD TG 406 animals should be scored with whole numbers only, and the results are therefore considered equivocal since it could be questioned if the scoring should be 1 or not.

Finally, one new study was included compared to the RAC evaluation in 2017. In this study, an *in vitro* transcriptomic and proteomic based approach predicted that glyphosate is not a skin sensitiser (CA 5.2.6/023, 2020).

RAC concludes that based on the negative results from the GPMT and LLNA tests, **no classification for skin sensitisation is warranted**.

RAC evaluation of specific target organ toxicity – repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The DS summarised a wide range of studies, including eight 28d oral studies in rats, mice and dogs, eleven 90d oral studies in rats, three 90d oral studies in mice, thirteen 90d and 1-year oral studies in dogs (table 46 in the CLH dossier). The DS also requested the Applicant to provide a NTP study (1992) including a 90d study in rats and mice. Five dermal studies in rats and rabbits and one inhalation study in rats were assessed. In addition, the DS included seven developmental toxicity studies in rabbits due to the maternal mortality observed in this species. Furthermore, one *in vitro* study with HepaRG cell culture and one *in vivo* study in mice exposed intranasally to filter extracts from "farm air" samples obtained during or after spraying with glyphosate were included, although not considered relevant for classification. No human data were included in the assessment. The DS noted that classification for STOT RE in Category 2 was proposed by the previous DS (Germany) in the CLH dossier from 2016. This classification was based on the maternal toxicity observed in the developmental toxicity studies in rabbits. However, in 2017 RAC concluded that STOT RE classification was not justified based on a weight of evidence approach. In the current assessment, the DS proposed to align with the previous opinion by RAC from 2017 and concluded that no classification for STOT RE is needed as no new findings or new evidence was provided that was considered relevant for a classification for STOT RE.

Comments received during consultation

Comments no. 209 - 231 submitted during consultation were related to the hazard class STOT RE. Eighteen comments supported the DS proposal for no classification for STOT RE. These comments were provided by Industry Trade Organisations, civil society NGOs and individuals. Comment no. 227 from the GRG (Company-Manufacturer) also supported the proposed no classification from the DS.

Comment no. 229 raised the issue of neurotoxicity in the RAR section 2.6.7 and pointed out that several publications indicate that glyphosate-based herbicides and glyphosate alone can alter the concentrations of several neurotransmitters in various regions of the brain in rodents. The DS responded that the studies mentioned in that comment had been considered in the process but noted that the studies were either considered to be non-relevant for the risk assessment or reliable with restrictions due to methodological limitations. The DS noted that OECD guideline compliant neurotoxicity studies with glyphosate did not indicate a neurotoxic potential and pointed out that all available information was taken into account in a weight of evidence assessment to determine the neurotoxic potential of glyphosate.

Assessment and comparison with the classification criteria

The DS included summaries of short-term studies, non-cancer effects in long-term studies, neurotoxicity studies and data on maternal toxicity from developmental toxicity studies in rabbits in their evaluation of STOT RE. The developmental toxicity studies in rabbits are included since they showed maternal mortality occurring in this species. Regarding human information, no data were available according to the DS.

Short term oral toxicity

Glyphosate was tested in several short-term and long-term oral studies using rats, dogs and mice. In addition, some studies by the dermal route using rats and rabbits were also included in the CLH dossier as well as one inhalation study in rats.

Eight 28d studies in rats, mice and dogs were assessed by the DS. No effects were observed within the guidance values for a classification for STOT RE in any of the six studies considered as "acceptable but with restrictions" by the DS. The NOAELs observed in these studies were above 1000 mg/kg bw/d; however, for several of the studies no NOAEL could be derived as they were designed as range finding studies with limited reporting.

The mechanistic study (non-guideline, non-GLP) by Gao *et al.* (2019) was considered as reliable with restrictions by the DS and it investigated the effects of glyphosate on renal proximal tubule cells *in vitro* and *in vivo*. The *in vitro* part of the study showed that glyphosate (as monoisopropylamine salt solution (40% w/w in water)) reduced cell viability, increased the incidence of apoptotic cells with an increase in the expression of apoptosis-related proteins, increased oxidative stress in a concentration related manner, increased the expression of the N-methyl-D-aspartate (NMDA) receptor and increased the Ca^{2+} influx. In the *in vivo* part of the study, kidney histopathology revealed exfoliation of renal tubular cells in the ICR male mice treated with glyphosate at 400 mg/kg bw/d for 28 days. Also, upregulation of apoptosis and NMDAR1 exposure in the proximal tubule epithelium and an imbalance of oxidant/antioxidant balance were observed. Based on this mechanistic study, the authors postulated that glyphosate could affect renal tubule epithelial cells via the NMDAR1/$[Ca^{2+}]$i/ROS pathway (ROS: reactive oxygen species).

The study by Tang *et al.* (2017) (non-GLP, non-guideline) was considered as supportive by the DS (purity of test substance unknown, only eight animals per dose group, only males) and investigated the effects of glyphosate on liver function and induction of pathological changes in ion levels and oxidative stress in hepatic tissue in rats. Sprague-Dawley rats were treated orally by gavage with 0, 5, 50, or 500 mg/kg bw/d of glyphosate (purity not reported) for 35 days. Adverse effects were noted at 50 and 500 mg/kg bw/d and comprised reduced body weight gain at both dose levels, and decreased absolute and relative spleen weight at 500 mg/kg bw/d. Furthermore, signs of oxidative stress, upregulation of liver inflammatory genes and upregulation of genes related to lipid metabolism were noted at 50 mg/kg bw/d and above, but effects were mainly slight and/or clinical relevance of these findings is lacking.

Tang *et al.* (2020) (non-GLP, non-guideline) was provided under the targeted consultation and studied the effects of glyphosate on the small intestine in male adult rats exposed to glyphosate by oral gavage to 0, 5, 50 or 500 mg/kg bw/d for 35 days (8 rats/group). Indicators of oxidative stress, ion concentrations and inflammatory responses were assessed in different segments of the small intestine (duodenum, jejunum and ileum). The results showed that glyphosate exposure decreased the ratio of villus height to crypt depth in the duodenum and jejunum. Decreased activity of antioxidant enzymes (T-SOD, GSH, GSH-Px) with most pronounced effects in the jejunum and ileum, and elevated malondialdehyde (MDA) content (only in the ileum) were reported. Furthermore, the mRNA expression levels of IL-1β, IL-6, TNF-α, MAPK3, NF-kB, and Caspase-3 were increased after glyphosate exposure, however, not consistently between the three segments of the small intestine. In addition, the concentrations of Fe, Cu, Zn and Mg were decreased in the duodenum and jejunum and increased in the ileum.

Eleven 90d oral studies in rats demonstrated overall low toxicity of glyphosate in different rat strains (table 46, CLH dossier) as well as the 1992 NTP study. Several of the studies showed no adverse effects up to 1000 mg/kg bw/d. One study (CA 5.3.2/003, 1996) reported a NOAEL of 79 mg/kg bw/d, with a corresponding LOAEL of 730 mg/kg bw/d. It is, however, noted that the large dose spacing (factor of 10 between low and mid dose) could have influenced the low NOAEL.

Observations of soft stools and diarrhoea together with occasionally reduced body weight gain indicated that glyphosate caused some irritation to the gastrointestinal (GI) tract in the high dose group of 3706 mg/kg bw/d. Blood or haemoglobin in the urine and a decrease in urine pH was also observed. However, all these effects were observed at doses well above the guidance values for classification for STOT RE (STOT RE 1: C ≤ 10 mg/kg bw/d and STOT RE 2: 10 < C ≤ 100 mg/kg bw/d). Another study (CA 5.3.2/011, 1991) showed a statistically significantly increased incidence of parotid cellular alterations in the salivary gland, described as deep basophilic staining and enlargement of cytoplasm, in both sexes at 1000 mg/kg bw/d. The incidence was 100% in males (compared to 30% in controls) and 90% in females (compared to 20% in controls). The severity grade was described as severe in males and moderate in females. This finding was also statistically significantly increased in the mid dose group (300 mg/kg bw/d). The incidence was similar to the high dose group; however, the severity grade was described as very mild to mild in the mid dose group. In the low dose group (30 mg/kg bw/d), the increased incidence of parotid cellular alteration was statistically significant only for females. The incidence was 70% in males (compared to 30% in control) and 80% in females (compared to 20% in control) and the severity grade of findings was minor (mostly very mild). In the NTP (1992) 90d study in rats, statistically significant increases in morphological changes were also reported in the parotid and submandibular salivary glands (combined) starting in males and females as minimal in the low dose group (205 mg/kg bw/d in males and 213 mg/kg bw/d in females) with increases in number of animals affected and in severity up to the highest dose tested (3393 mg/kg bw/d in males and females). Overall, the effect on the salivary gland is considered treatment-related, and human relevance cannot be excluded. However, RAC considers that this finding is not sufficient for a classification as STOT RE since these effects were only minor at doses within the guidance values for classification for STOT RE (STOT RE 1: C ≤ 10 mg/kg bw/d and STOT RE 2: 10 < C ≤ 100 mg/kg bw/d) and were not reported in the other short-term studies in rats. RAC notes that in a 1-year study with rats (CA 5.5/006, 1996) effects on parotid salivary gland starting at 560 mg/kg bw/d in male rats were observed.

Four 90d studies, one 6-month study and five 1-year studies (table 46, CLH dossier), showed that dogs have a similar sensitivity to glyphosate to that observed in the rat. In the 90d studies, the LOAEL values observed started at 250 mg/kg bw/d. In the study showing the lowest LOAEL (CA 5.3.2/021-024, 1999), Beagle dogs received glyphosate (purity > 95%) at dietary dose levels of 0, 200, 2000 or 10000 ppm (corresponding to 5.2, 54.2 or 252.4 mg/kg bw/d in males and 5.4, 52.8 and 252.7 mg/kg bw/d in females), 4 males/4 females per dose level, for 90 days. Decreased food consumption was observed in both sexes in the second week of treatment (-47% in males and -37% in females). Further, increased levels of gamma-glutamyl transferase (GGT, +171% in males and +91% in females after 45 days) and alkaline phosphatase (ALP, +129% in males after 45 days) were also observed in high dose animals. In addition, higher levels of total bilirubin were seen at all dose levels (+98% in males and +79% in females after 90 days); however, as no effects were seen on the liver, only the increased levels of bilirubin at the top dose were considered adverse, as these were accompanied by increased GGT and ALP levels. Further, in the 13-week dog study (CA 5.3.2/020, 2007) animals showed severe signs of toxicity at 1000 mg/kg bw/d, including liquid/soft faeces, dehydration, thin appearance, vomiting and pallor, reduced feed consumption and effects on body weight. The maximum tolerable dose (MTD) was clearly exceeded in this study. In a 6-month study with six Beagle dogs/dose/sex (CA 5.3.2/029, 1983) exposed to daily doses of 0, 10, 60 or 300 mg/kg bw/d, a decreased body weight was observed in males (-13%) at the end of the study in the high dose group. The five 1-year studies with Beagle dogs reported LOAEL values from 500 mg/kg bw/d.

Three 90d oral studies in mice showed that the toxicity of glyphosate was similar to that reported for rats (table 46, CLH dossier). The first 90d study showed a NOAEL of 1221 mg/kg bw/d (CA 5.3.2/017, 1995). The second study (CA 5.3.2/018, 1991) reported no effects at the highest dose level of 4500 mg/kg bw/d. The parotid gland was not examined in the studies CA 5.3.2/017

(1995) and CA 5.3.2/018 (1991); however, no effects were noted for either the sublingual or submaxillary glands that were examined in these two studies. The third study (CA 5.3.2/019, 1979) in CD-1 mice showed no treatment related adverse effects at 5000 and 10000 ppm (up to 1867 mg/kg bw/d in males and 2734 mg/kg bw/d in females). At the top dose of 50000 ppm (9707 mg/kg bw/d in males and 14858 mg/kg bw/d in females) bodyweight gain was decreased by up to 24% in males and 18% in females. No other adverse effects were observed. In the NTP (1992) 90d study in mice, a statistically significant increase in morphological changes were reported in the parotid salivary glands starting in males and females as minimal from 1065 mg/kg bw/d in males and 1411 mg/kg bw/d in females with a dose-dependent increase in number of animals affected and in severity up to the highest dose tested (10780 mg/kg bw/d in males and 11977 mg/kg bw/d in females). Overall, these studies consistently showed no adverse effects in mice within the guidance values relevant for a STOT RE classification.

In conclusion, the short-term studies in rats, mice and dogs did not show effects relevant for classification at doses below the guidance values for STOT RE (STOT RE 1: C ≤ 10 mg/kg bw/d and STOT RE 2: 10 < C ≤ 100 mg/kg bw/d for 90d studies).

Short term dermal studies

Repeated exposure to glyphosate through the dermal route has been investigated in several 21/28d studies in rats and rabbits (table 46, CLH dossier); however, none of the studies showed any effects at doses relevant for a classification for STOT RE.

In a 21d dermal toxicity study (CA 5.3.3/001, 1996), groups of five male and five female Wistar rats received 6h dermal applications of 0, 250, 500 or 1000 mg glyphosate acid/kg bw/d. No effects indicating systemic toxicity and no dermal irritation occurred at any dose level. In another 21-d dermal toxicity study (CA 5.3.3/003, 1993) there were no systemic effects observed in animals dermally treated at 1000 mg/kg bw/d for three weeks. However, mild irritant effects (erythema and desquamation) were noted at the dosing site (3/5 males and 5/5 females).

In rabbits, repeated dermal application to male and female New Zealand Whites (NZW) at doses of 0, 500, 1000 or 2000 mg/kg bw/d for a 6h period on five consecutive days per week over four weeks (CA 5.3.3/004, 1994) showed no treatment-related signs of systemic toxicity at any dose level. Local effects were limited to a very slight erythema noted in one high dose male and one low dose female. Two additional 21d dermal toxicity studies in rabbits, both considered unacceptable, did not show any effects relevant for a classification for STOT RE.

Short term inhalation study

Repeated exposure to glyphosate through the inhalation route has been investigated in one 14-d inhalation study in rats; however, the DS considered this pre-GLP and non-guideline study as not acceptable due to serious reporting deficiencies, e.g., absence of statistical analysis, and purity and batch number of the test substance not reported. Up to the highest concentration tested of approx. 3.8 mg/L air (mean measured concentration) repeated inhalation exposure of Wistar rats to an aerosol containing glyphosate did not lead to any local (respiratory) or systemic toxicity (CA 5.3.3/009, 1985).

Kumar *et al.* (2014) studied the mechanism of airway inflammation following intranasal exposure to 0, 0.1, 1 or 100 µg glyphosate in female mice (8 mice/group): C57BL/6 WT and-TLR4-/- mice and BALB/c female WT mice and IL-13-/- mice daily for 7 days or 3 times/week for 3 weeks. The study was considered by the DS as acceptable with restrictions. The cellular response, humoral response and lung function of the mice were assessed. Exposure to 1 or 100 µg glyphosate resulted in increased total cell count/lung, as well as eosinophil and neutrophil counts compared to controls in WT mice, however, without a clear dose-response. No changes in the number of mast cells were reported. Further, exposure to glyphosate induced pulmonary IL-13-dependent inflammation and promoted Th2 type cytokines, but not IL-4. No effect was seen at 0.1 µg. IL-

33 and TSLP (involved in airway inflammation) were increased in the respiratory epithelium of glyphosate-treated wild-type mice, and inflammation was confirmed by histological examination. The study concluded that exposure to glyphosate induced minor exacerbation of immune response in WT female mice. RAC notes that glyphosate was administered (30 µL) to the nose of anesthetised mice in order to aspirate the solution, and it is unclear how aspiration of glyphosate is related to an exposure to glyphosate via inhalation.

In vitro study

HepaRG human liver cell culture was used to investigate the effects of glyphosate (0, 0.06, 6 or 600 µM) on the transcriptome and metabolome profile (Mesnage *et al.*, 2018). This study was not included in the RAC opinion from 2017 and was considered to be reliable with restriction by the DS. Glyphosate was weakly toxic and induced small changes in transcriptome profiles. The metabolomics analysis of HepaRG cells exposed to 0.06 µM glyphosate induced a statistically significant decreased level of long chain fatty acids and polyunsaturated fatty acids. At 6 and 600 µM glyphosate, lower lipid levels were also observed, without reaching statistical significance. While the study gives some indication of a slight potential effect of glyphosate on transcriptome profile alterations in HepaRG human liver cells *in vitro*, it does not provide information on a potential adverse effect *in vivo*.

Neurotoxicity studies

The DS included two 90d sub-chronic neurotoxicity studies in their assessment of STOT RE. Overall these two studies did not show any significant or severe toxicity below the oral guidance values and no classification for STOT RE is warranted based on these studies.

In the first study according to OECD TG 424 (CA 5.7.1/002, 2006, acceptable according to the DS), groups of 10 male and 10 female Sprague-Dawley rats were fed diets containing 0, 1000, 5000 or 20000 ppm glyphosate (corresponding to dose 0, 77, 395, or 1499 mg/kg bw/d in males and 0, 78, 404, or 1555 mg/kg bw/d in females) for 90 days. The only adverse effect observed was a decrease in body weight (-12%), body weight gain (-15%) and food consumption (up to -17%) in high dose males. No treatment-related changes in neurological parameters were observed.

In a second study according to OECD TG 424 (CA 5.7.1/003, 1996, according to the DS acceptable with restrictions), groups of 12 male and 12 female Alpk:APfSD (Wistar-derived) rats were fed diets containing 0, 2000, 8000 or 20000 ppm glyphosate acid for 13 weeks. The only adverse effect observed was decreased body weight gain (-12%) and food efficiency in high dose males. No treatment related changes in neurological parameters were observed.

In addition, several publications on neurotoxicity were evaluated. Martinez *et al.* (2019) evaluated the effect of glyphosate and its metabolite AMPA on the blood-brain barrier *in vitro*. Overall, the study does not indicate a neurotoxic potential for glyphosate or AMAP which is in line with the guideline studies available. Martinez *et al.* (2018) observed an effect of glyphosate on neurotransmitter levels in rat brain regions after oral dosing by gavage at 35, 75, 150, 800 mg/kg bw/d for 6 days. However, the study was a non-guideline *in vivo* study with no concurrent positive control and no positive and negative historical control data (HCD) included, and it is therefore difficult to interpret the biological relevance of the observed changes. Chorfa *et al.* (2013) evaluated the effect of glyphosate on α-syn levels in human neuroblastoma (SH-SY5Y) and melanoma (SK-MEL-2) cell lines. Glyphosate did not have any impact on the endpoints measured in this study. Ait-Bali *et al.* (2020) investigated behavioural, neurochemical, and molecular changes after pre- and post-natal exposure of mice to a Roundup formulation (glyphosate concentration: 360 g/L as isopropylamine salt 486 g/L). It is noted that any effect of the co-formulant(s) in Roundup cannot be excluded. In this study, groups of 10 female Swiss mice received Roundup by gavage at concentrations of 250 or 500 mg/kg bw/d from gestational day 0 (GD0) to postnatal day 21 (PND21). At PND60 effects at the behavioural, neurochemical and

molecular levels were examined. The results show that pre- and neonatal exposure to the Roundup formulation impairs fertility and reproduction parameters as well as maternal behaviour of exposed mothers. In offspring, exposed animals show a delay in innate reflexes and a deficit in motor development. At the adult age, exposed animals showed a decrease of locomotor activity, sociability, learning and short- and long-term memory associated with alterations of cholinergic and dopaminergic systems. The formulation also activated microglia and astrocytes, sign of neuroinflammation event in the medial prefrontal cortex and hippocampus. At the molecular level, a downregulation of BDNF expression and an upregulation of TrkB, NR1 subunit of NMDA receptor as well as TNFα were found. The study is considered as supplementary by the DS.

Long-term studies (non-neoplastic effects)

A large number of long-term studies have been performed in rats and mice (table 53, CLH dossier). Neoplastic effects are further described in the carcinogenicity section. Occurrence of non-neoplastic effects in these studies can be relevant for classification for STOT RE. However, none of the long-term studies presented in the CLH dossier reported effects at dose levels relevant for classification with STOT RE (2-year study: STOT RE 1: C ≤ 2.5 mg/kg bw/d and STOT RE 2: 2.5 < C ≤ 25 mg/kg bw/d). A 1-year study with rats (CA 5.5/006, 1996) observed effects on body weight, food consumption, food efficiency, alkaline phosphatase activity and focal basophilia of acinar cells of parotid salivary gland starting at 560 mg/kg bw/d in male rats. In at least three of the 2-year studies in rats and mice effects were seen starting at 300 - 400 mg/kg bw/d, whereas the LOAEL was much higher in the remaining studies.

Maternal toxicity in developmental studies in rabbits

Findings from developmental toxicity studies can also be of relevance for classification for STOT RE according to the CLP Regulation (Annex I, 3.9.2.5). Thus, the use of the rabbit developmental studies for the assessment of STOT RE is considered justified by RAC.

A wide range of studies are available; these include multi-generation studies in rats and developmental toxicity studies in rats and rabbits. The 2-generation studies with rats showed treatment related findings at very high doses, with reported NOAELs in the range of 200 - 1000 mg/kg bw/d. The developmental studies in rats showed NOAELs for maternal toxicity starting at 300 mg/kg bw/d; however, for most studies, no effects on maternal toxicity were seen up to the limit dose for reproductive toxicity (1000 mg/kg bw/d; OECD TG 414).

However, rabbits seem to be a much more sensitive species for effects arising from glyphosate exposure. Findings, including maternal deaths, are summarised in the table below.

Table: *Rabbit maternal mortality and toxicity from developmental toxicity studies with glyphosate.*

Study, purity, strain, duration, dose levels, female rabbits per group	Premature deaths and cause of deaths*	Further maternal effects	Maternal NOAEL / LOAEL (mg/kg bw/d) Corrected Guidance values**
CA 5.6.2/019, 1980; 98.7% Dutch Belted rabbit GD6-27 Gavage 0, 75, 175, 350 mg/kg bw/d 16 female rabbits per group (17 in high dose group)	Found dead: 1, 2, 10 at 75, 175 and 350 mg/kg bw/d, respectively. At 350 mg/kg bw/d 1 animal died prior to treatment and was replaced. Out of these, 1, 1 and 3 deaths at 75, 175 and 350 mg/kg bw/d, respectively, were not regarded as being substance related (pneumonia, respiratory disease, enteritis or gastroenteritis). Cause of death could not be determined for remaining 8 animals. First death: GD14 (350 mg/kg bw/d)	Soft stool & diarrhoea (noted in all dose groups but increased compared to control from 175 mg/kg bw/d). No treatment related effect on maternal bw and bw gain in female rabbits that survived to scheduled time.	75/175 Corrected guidance values STOT RE 1: ~43 STOT RE 2: ~430

Study, purity, strain, duration, dose levels, female rabbits per group	Premature deaths and cause of deaths*	Further maternal effects	Maternal NOAEL / LOAEL (mg/kg bw/d) Corrected Guidance values**
Study considered as supportive information in RAR.	Further deaths: day 17, 18, 21 (350 mg/kg bw/d); 22, 25 (175 mg/kg bw/d); 26 (75 mg/kg bw/d). Abortions: 2 (GD22), 1 (GD27), 1 (GD23) were sacrificed after abortion at 0, 175 and 350 mg/kg bw/d		
CA 5.6.2/014, 1991; 98.6% NZW rabbit GD7-19 Gavage 0, 50, 150, 450 mg/kg bw/d 16 - 20 female rabbits per group Study considered acceptable in RAR.	Found dead: One premature death at 450 mg/kg bw/d on GD20. Mortality occurred after cessation of treatment and signs of abortion GD19, signs of GI disturbance, severe reduction in food consumption and bodyweight loss. Two other deaths were unrelated to the treatment (broken hindleg at 450 mg/kg bw/d and congenital abnormality in control group). Abortions: 1 at 50 mg/kg bw/d (whole litter), 1 at 150 mg/kg bw/d (aborted 1 of 9 foetuses, remaining litter values are included in assessment).	Soft/liquid stool (2, 5, 13 animals at 50, 150 and 450 mg/kg bw/d) (dose-related increase). Reduced food consumption compared to the control (12% day 11 - 19 at 150 mg/kg bw/d and 6 - 17% day 7-19 at 450 mg/kg bw/d. A slight reduction in bw gain from GD11 to termination at 150 and 450 mg/kg bw/d.	50/150 Corrected guidance values STOT RE 1: ~75 STOT RE 2: ~750
CA 5.6.2/012-013, 1993; 96.8% NZW rabbit GD6-18 gavage 0, 20, 100, 500 mg/kg bw/d 15 - 17 female rabbits per group in treated groups, 26 in control Study considered supplementary in RAR.	Found dead: Two premature deaths (control) due to mis-gavage. Four (100 mg/kg bw/d), 8 (500 mg/kg bw/d),) died from treatment; however, several of these animals were shown to have pathological changes in the lungs. First death: GD7 (2x 100 mg/kg bw/d; 1x 500 mg/kg bw/d) Further deaths: day 9, 18 (100 mg/kg bw/d) 11, 14, 15, 18, 19 (500 mg/kg bw/d) Abortions: No information regarding abortions.	At 500 mg/kg bw/d: Soft/liquid stool (stat. sign). Significantly reduced food consumption (31%, GD6-19). Significantly reduced maternal body weight and body weight gain. Toxicity symptoms involving rales, dyspnoea and weakness.	20/100 Corrected guidance values STOT RE 1: ~75 STOT RE 2: ~750
CA 6.5.2/011, 1995; 97.56% Japanese White rabbit (Kbl:JW) GD6-18 Gavage 0, 10, 100, 300 mg/kg bw/d 18 female rabbits per group Study considered acceptable in RAR.	Found dead: One dead at 300 mg/kg bw/d (no clinical signs), day 20. Abortions: Two at 10 mg/kg bw/d (day 20, premature delivery day 27), 2 at 300 mg/kg bw/d (day 26, premature delivery day 27).	Four animals showed loose stool in the high dose group. Loose stools were also seen in two control animals and in one animal in the low dose group. No significant effect on food consumption and body weight.	100/300 Corrected guidance values STOT RE 1: ~75 STOT RE 2: ~750
CA 5.6.2/010, 1996; 95.3% NZW rabbit GD7-19	Found dead: Two at 400 mg/kg bw/d (day 19 and 20). One found dead, one killed in extremis.	Scours. At 400 mg/kg bw/d stat. sign. ↓ in food consumption from GD10-19 and ↓ bw gain from GD9-29 stat. sign. from day 13.	50/200 Corrected guidance values STOT RE 1: ~75

Study, purity, strain, duration, dose levels, female rabbits per group	Premature deaths and cause of deaths*	Further maternal effects	Maternal NOAEL / LOAEL (mg/kg bw/d) Corrected Guidance values**
Gavage 0, 50, 200, 400 mg/kg bw/d 18 female rabbits per group Study considered acceptable in RAR.	One in control found dead after dosing. One at 200 mg/kg bw/d found dead day 16 (mal-dosing). Abortions: The animal killed in extremis day 20 showed signs of abortion.	Vaginal bleeding and blood on tray were noted for 1 animal at 200 mg/kg bw/d.	STOT RE 2: ~750
CA 5.6.2/009, 1996; 95.6% NZW rabbit GD8-20 Gavage 0, 100, 175, 300 mg/kg bw/d 20 female rabbits per group Study considered acceptable in RAR.	Abortions: One in control (day 30), 2 at 100 mg/kg bw/d (day 19 and 25), 1 at 175 mg/kg bw/d (day 22), 2 at 300 mg/kg bw/d (day 23 and 24). One at 175 mg/kg/bw/d killed for humane reasons (day 23) following bw loss and reduced food consumption.	Diarrhoea, ↓ food consumption accompanied by a stat. sign. ↓ bw gain in high dose group from GD17-26.	100/175 Corrected guidance values STOT RE 1: ~75 STOT RE 2: ~750
CA 5.6.2/018 1980; 100/technical glyphosate Pilot study Pre-guideline/GLP Dutch Belted rabbit GD6-27 Gavage 0, 125, 250, 500, 1250 and 2500 mg/kg bw/d 5/females pet group Study considered supportive in RAR.	Mortality: 500 mg/kg bw/d: 4/5 (GD15-22) 1250 mg/kg bw/d: 5/5 (GD10, 11) 2500 mg/kg bw/d: 5/5 (GD9, 10), one attributed to gavage error. Abortions: 1/5 at 500 mg /kg bw/d, GD26	The body weight gain was reduced in animals administered doses of 500 mg/kg bw/d and higher but due to the high mortality, only data for animals up not 250 mg/kg bw/d are available for the entire study period.	Not applicable Corrected guidance values STOT RE 1: ~43 STOT RE 2: ~430

* There is a lack of consistency between the studies in how an animal that aborted is "labelled" i.e., it was either described as "killed in extremis" or "killed due to abortion" and sometimes an animal that was "found dead" had shown signs of abortion. However, in many cases all these "labels" can at least partly be viewed as just representing different expression of the same toxicity.

** CLP 3.9.2.9.8: "Guidance values are intended only for guidance purposes i.e., to be used in a weight of evidence analysis. They are not intended as strict demarcation values". In rabbits the perturbed digestion alters the absorption of glyphosate thus influencing the actual dose absorbed from the GI tract.

Six out of the seven studies presented in the table above showed premature maternal deaths. These maternal deaths cannot be considered to reflect an acutely toxic effect since they occurred after several days of treatment. In three studies (CA 5.6.2/019, 1980; CA 5.6.2/012-013, 1993; CA 5.6.2/010, 1996) reporting premature death, the cause of death for some animals was suggested to be due to mis-gavage. The presence of premature deaths was observed in female rabbits along with decreased food consumption and reduced bw gain in five of the six studies. However, decreased food consumption and reduced bw gain were also reported in female rabbits

without premature death at similar doses of glyphosate to those administered in the studies with premature death. Therefore, the premature death reported is not considered to be only related to decreased food consumption and reduced bw gain. Soft/liquid stool and diarrhoea was also a consistent feature reported in most of the rabbit developmental toxicity studies indicating a local irritating effect of glyphosate in the GI tract. It was reported in female rabbits from studies with both a high level of premature deaths and in studies with none or low levels of maternal premature deaths. Therefore, a clear association between the premature maternal deaths and soft/liquid stool and diarrhoea cannot be established. Since in some of the studies the cause of some of the premature deaths was not clear (i.e., due to problems with the dosing technique or due to infections), and soft/liquid stool were also in some cases reported for controls, no clear association between premature death and these effects could be established. These clinical signs were also reported in some of the 2-generation and developmental toxicity studies in rats following repeated exposure to glyphosate without leading to death of the animals.

Caecotrophes are the material resulting from the fermentation of food in the rabbit caecum. They are nutrient-rich and are passed out of the body, like faeces, but are re-ingested by the animal so the nutrients can be absorbed. Several of these studies reported that the rabbits showed soft stools and/or diarrhoea. Maternal toxicity can be related to soft stools and diarrhoea because these effects may prevent the rabbits from eating their caecotrophs, often an essential, specialised digestive strategy for the recycling of caecal contents and the extraction of nutrients. However, studies of rabbits completely deprived of caecotrophs demonstrate that while caecotrophy is very important for normal growth, it is not always essential for survival (Robinson *et al.,* 1985; Phiny *et al.,* 2006). In the studies detailed above there is no information that the animals were not able to eat their caecotrophes. If the animals are ingesting their caecotrophes, one could anticipate that female rabbits will be exposed to un-metabolised glyphosate repeatedly since glyphosate is excreted unchanged via the faeces. Therefore, the recirculation of digestive material containing glyphosate will have an influence on the actual dose absorbed from the GI tract.

According to the CLP criteria, all available evidence, and effects relevant to human health, shall be taken into consideration in the classification process. This can include morbidity or death resulting from repeated or long-term exposure. The guidance values for classification in Category 1 for a 90d oral exposure study in rats is less than 10 mg/kg bw/d, and for a 28d study less than 30 mg/kg bw/d. The guidance value for classification in Category 2 is less than 100 mg/kg bw/d for a 90d oral exposure study, and less than 300 mg/kg bw/d for a 28d study. However, according to the CLP Regulation (Annex I, 3.9.2.9.8): "*Guidance values are intended only for guidance purposes i.e. to be used in a weight of evidence analysis. They are not intended as strict demarcation values*". There are no guidance values specified for oral exposure of rabbits, but RAC considers that the guidance values for rats might be used as part of a weight of evidence also for other species, including rabbits.

For the evaluation of the rabbit developmental toxicity studies in the table above, the findings at particular doses have been compared with guidance values corrected for the duration of the exposure (according to Haber's rule). It can be seen from the table that all five studies showed premature deaths within the corrected guidance values for classification with STOT RE 2. However, it is important to take into account that guidance values are only for guidance purposes and that the perturbed digestion in the female rabbits may alter the absorption of glyphosate, thus, influencing the actual dose absorbed from the GI tract. Therefore, the use of Haber's rule to correct the guidance values in these studies includes uncertainties and the results should be used with caution.

In the CA 5.6.2/012-013 (1993) study, with a high level of premature deaths, two premature deaths were also reported in the control group and were confirmed to be due to mis- or mal-dosing. In the RAR (2021), part B6.6, some doubts were also raised relating to the four deaths

reported at 100 mg/kg bw/d since there were no signs of toxicity at this dose level. In the other rabbit developmental toxicity studies, no deaths were reported at similar dose levels, further contributing to doubts over the cause of the deaths reported at this dose level in the CA 5.6.2/012-013 (1993) study. In addition, at gross necropsy various findings were noted in the lung and trachea in the mid and high dose groups (100 and 300 mg/kg bw/d, respectively) in the female rabbits that died. In the high dose group microscopic examination showed that five out of eight female rabbits had lung lesions (emphysema, collapsed, pneumonic lesions, consolidated and congested) and in the mid dose group one out of four female rabbits that died had lung and trachea congestion and froth in the trachea suggesting that gavage errors could have contributed to some of the deaths reported at these dose levels.

In the study CA 5.6.2/019 (1980), 3/10 mortalities at 350 mg/kg bw/d, one mortality at 175 mg/kg bw/d and one mortality at 75 mg/kg bw/d were reported to be due to pneumonia, respiratory disease, enteritis, or gastroenteritis. Unfortunately, there was no necropsy report attached to the original study report and the cause of death for the remaining 7/10 animals in the high dose group and one animal at 175 mg/kg bw/d and one animal at 75 mg/kg bw/d were not reported with any degree of detail so it cannot be ascertained if it was substance related or not. Premature deaths were also reported in the CA 5.6.2/011 (1995), CA 5.6.2/010 (1996) and CA 5.6.2/014 (1991) studies at doses from 300 to 450 mg/kg bw/d without reporting of mis-dosing, all with a lower incidence of mortality than reported in the CA 5.6.2/019 (1980) and CA 5.6.2/012-013 (1993) studies. There are some uncertainties remaining related to the cause of the premature maternal deaths in the CA 5.6.2/012-013 (1993) and CA 5.6.2/019 (1980) studies, since it is not clear if the deaths were attributable to exposure to glyphosate, to mis-dosing or to infections (e.g., pneumonia, respiratory disease). Altogether, RAC considers that the premature maternal deaths reported in several rabbit developmental toxicity studies cannot be viewed as clear evidence of glyphosate toxicity following repeated exposure.

According to the CLP Regulation (Annex I, 3.9.2.9.7): "*Classification in Category 2 is applicable, when significant toxic effects observed in a 90day repeated dose study…are seen to occur within…*" a range of 10 < C ≤ 100 mg/kg bw/d via oral exposure in the rat. Applying Haber's rule for a study of shorter duration (28 days) allows for extrapolation of the guidance values to a range of 30 < C ≤ 300 mg/kg bw/d via the oral route. However, in this case the use of Haber's rule to correct the guidance values includes uncertainties and the results should be used with caution.

The DS proposed no classification for STOT RE based on the weight of evidence assessment by RAC from the previous assessment in the opinion from 2017, and further, on that no new evidence was provided for the current assessment. RAC is of the opinion that large doses of glyphosate are associated with severe maternal toxicity and death in female rabbits. However, as was noted in the CLH opinion in 2017 (reproduced in the current CLH dossier), the overall weight of evidence for classification for STOT RE is unconvincing due to the following reasons:

1. *Strictly, there are only 2 studies with deaths reported below the corrected guidance value, i.e. 4 female rabbits in the CA 5.6.2/012-013 (1993) study at 100 mg/kg bw/d and 8 female rabbits at 500 mg/kg bw/d, and 2 female rabbits in the CA 5.6.2/019 (1980) study at 175 mg/kg bw/d and 10 female rabbits at 350 mg/kg bw/d where several of the deaths in each study could be related to mal-gavage.*

2. *In the CA 5.6.2/012-013 (1993) study, pathological changes in the lungs were noted in one of the dead animals at 100 mg/kg bw/d and were suggestive of gavage errors. The remaining 3 decedents in the 100 mg/kg bw/d dose-group had no abnormalities and there were no reported clinical signs at this dose level. Five out of 8 the mortalities in the high dose group also displayed pathological changes suggestive of gavage errors. The remaining 3 decedents in the 500 mg/kg bw/d group had no abnormalities. Soft stool and diarrhoea were reported, however, a clear association with premature death cannot be established. There were also 2 mis-dosing in the concurrent controls. Overall, the frequent*

reporting of pathological findings in the lung suggestive of gavage errors raises concern regarding the technical skills in dosing via oral gavage and consequently also on the inclusion of this study in the assessment of substance induced mortality.

3. *In the CA 5.6.2/019 (1980) study, 1, 1 and 3 premature deaths at 75, 175 and 350 mg/kg bw/d, respectively, out of 1, 2 and 10 premature deaths at these dose levels were reported to be due to pneumonia, respiratory disease, enteritis or gastroenteritis; the remaining death was unexplained.*

4. *Five of the studies included in the table "Rabbit maternal mortality and toxicity from developmental studies with glyphosate" with dosing over the range 50 to 450 mg/kg bw/d did not reveal signs of an increased mortality as observed in the study by CA 5.6.2/012-013 (1993) and CA 5.6.2/019 (1980).*

5. *The majority of deaths were associated with high doses of glyphosate and the majority of deaths were associated with 2 studies where the cause of death is unclear.*

6. *The physiology of digestion in the rabbit is in some ways unique. In rabbits, caecotrophy ensures that substances predominantly excreted unchanged in the faeces such as glyphosate are readily available for repeated oral uptake and constitute a potentially significant oral dose relative to other species including humans. This possible recycling of glyphosate and increased exposure in rabbits might explain the particular sensitivity of this species while at the same time casting doubt over the relevance of oral dosing in rabbit studies for humans. However, there is a lack of information regarding whether the rabbits were able to eat their caecotrophes or not, and therefore it is not possible to have a clear picture of a possible recycling of glyphosate and consequently the actual dose absorbed from the GI tract, leading to uncertainties with using Haber's rule to correct the guidance value for a STOT RE classification in these studies.*

7. *Signs of digestive disturbances (soft/liquid stool and diarrhoea) were consistently reported in the rabbit studies (but also in rats at much higher doses). However, a clear association with premature maternal death cannot be established. The fact that the female rabbits appear to be uniquely sensitive compared to rodent dams further support the caecotrophy hypothesis and weakens the argument for classification in this case.*

Furthermore, an in-depth analysis of all the data from both the short-term and long-term toxicity studies only shows effects at high dose levels exceeding the extrapolated guidance values relevant for a classification with STOT RE.

The possibility that mortality in female rabbits could lead to a classification of glyphosate for STOT RE 2 has also been discussed in the current CLH dossier. According to the CLP Regulation (Annex I, 3.9.2.7.3), morbidity or death resulting from repeated or long-term exposure can be taken into account for classification as STOT RE. However, the CLP Regulation further states that "*Morbidity or death may result from repeated exposure, even to relatively low doses/concentrations, due to bioaccumulation of the substance or its metabolites, and/or due to the overwhelming of the de-toxification process by repeated exposure to the substance or its metabolites*".

In summary, the conclusion of RAC on this hazard class is the same as in the CLH opinion on glyphosate in 2017: *Following exposure to glyphosate, mortality in rabbits is considered to either be related to mis-dosing, infections or diarrhoea and the possible mechanism of caecotrophy and recycling of glyphosate. No mortalities were recorded in the rat studies. In addition, bioaccumulation and over-whelming of detoxification mechanisms by repeated exposure as a mechanism of toxicity is not likely for glyphosate*.

RAC notes that from the short-term and long-term studies in rats, mice and dogs, no effects relevant for classification at doses below the relevant guidance values for classification for STOT RE was reported. Further, two 90d sub-chronic neurotoxicity studies and several publications on neurotoxicity did not show any significant or severe toxicity below the guidance values.

Based on a weight of evidence approach and with a review and due considerations of all data from the short-, long-term, reproductive and rabbit developmental studies, **RAC concludes that STOT RE classification is not warranted for glyphosate**.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

The DS summarised numerous *in vitro* studies with glyphosate, including standard bacterial assays and mammalian cell gene mutation tests, which consistently gave negative results (table 49 in the CLH dossier). The DS also noted that the majority of the *in vitro* chromosomal aberration tests and micronucleus tests were negative, and that in particular, all of the studies performed under GLP conditions resulted in negative findings. No evidence of chromosome aberrations was obtained in 11 guideline-compliant *in vivo* micronucleus assays or chromosome aberration studies in which the bone marrow of either mice or rats was examined after oral or intraperitoneal application (table 50 in the CLH dossier).

The DS also noted that in published studies with methodological limitations, the results were contradictory and that in most of these studies, relatively low dose levels were employed, and the intraperitoneal route was used "*which does not properly reflect the human exposure*" according to the DS.

Evidence of exposure to glyphosate was based on the affinity of glyphosate to bone marrow as shown in the toxicokinetic studies, by the occasional observation of bone marrow toxicity in the tests themselves and by the occurrence of hypoplasia in bone marrow in a long-term study in rats (at a very high dose).

Positive results were observed for induction of sister chromatid exchange (SCE) and DNA strand breaks (Comet assay) but a negative result in a study investigating induction of DNA repair (unscheduled DNA synthesis; UDS).

Based on a weight of evidence determination, the DS proposed no classification for germ cell mutagenicity.

Comments received during consultation

Comments no. 54 - 81 submitted during the consultation were related to the hazard class germ cell mutagenicity.

A total of 19 comments supported no classification for germ cell mutagenicity. These comments were provided by Downstream Users, Manufacturers, an Industry and Trade Organisation, a National civil society NGO, individuals, a Member State and a National Authority. A further six comments supported a classification for germ cell mutagenicity. These comments were provided by International and National civil society NGOs, an Academic institution and individuals.

Comments no. 56, 57 and 66 claimed that the assessment does not include all relevant publications on the genotoxic potential of glyphosate from the peer-reviewed literature. They added that key studies such as the Comet assay (in tissues other than the bone marrow e.g., liver and kidney) or Transgenic rodent (TGR) somatic and germ cell gene mutation assays are missing. The comments also pointed out that non-mammalian models such as fish, insects, worms, plants and crustaceans are not included in the genotoxicity assessment, and those obtained with glyphosate-based formulations would better reflect the reality of exposure in humans. In addition, the comments included a re-evaluation of the reliability of the mutagenicity

studies included in the CLH dossier. The DS responded that regarding the literature search, this was performed according to the relevant EFSA Guidance document (EFSA, 2011). All studies, including those from the public literature and studies submitted by the applicant, were assessed for their relevance and reliability, using EU agreed assessment points. Furthermore, the DS noted that the *in vitro* Comet assays found in the literature were indeed assessed and included in the CLH dossier, noting that there is no OECD test guideline for an *in vitro* Comet Assay. The DS pointed out that such studies in the public literature were not generally designed to follow OECD test guidelines. However, OECD test guidelines ensure that there are internationally agreed testing and validity criteria which standardise studies and ensure reliability, allowing mutual recognition of data. As regards the use of non-mammalian models, the DS noted that for the evaluation of glyphosate, Regulation (EC) 1107/2009 (Plant Protection Product (PPP) Regulation) and data requirements of Regulation (EC) 283/2013 apply, which specifically describe which studies should be conducted, and that these do not describe the use of tests on aquatic species to address genotoxicity. In addition, currently in the CLP Regulation no specific guidance is given on this point.

Comments no. 59 and 60 and 61 raised serious concerns about the statistical procedures, scientific quality, and therefore reliability, of the genotoxicity and mutagenicity studies submitted to the EU by glyphosate manufacturers. The DS responded that the authors' conclusions were compared to the previous evaluation report for glyphosate (EFSA, 2015) and not the current assessment. The current conclusions differ somewhat from the conclusions taken in the previous renewal evaluation (EFSA, 2015) with 11 additional studies included in the current renewal assessment. As regards the statistics used, the DS noted that in the renewal report (EFSA, 2015) they evaluated all genotoxicity studies and compared the methods used to current OECD test guidelines. Deviations and their influence on the reliability of the studies were noted also in relation to the statistical methods used. The DS pointed out that all relevant available information was taken into account in a weight of evidence assessment to determine the germ cell mutagenic potential of glyphosate.

Assessment and comparison with the classification criteria

RAC based its assessment of germ cell mutagenicity (as with all hazard classes) on the information supplied by the DS and that received through the consultation.

Glyphosate has been tested in a wide range of genotoxicity and mutagenicity assays. All relevant genotoxicity and mutagenicity studies included by the DS (tables 49, 50, 51 and 52 in the CLH dossier) have been considered and both guideline- and non-guideline studies form the basis of the current RAC mutagenicity assessment. In addition to the acceptable and supportive studies included in tables 49, 50, 51 and 52 in the CLH dossier, RAC notes that the DS also included in tables 49, 50 and 52 studies which were not acceptable or of low reliability for *in vitro* and *in vivo* genotoxicity and mutagenicity following exposure to glyphosate. These studies were not included in the overall weight of evidence assessment for germ cell mutagenicity due to factors such as sufficiently high dose levels not having been tested, the purity of the test substance, appropriate controls not included, reporting deficiencies and because cytotoxicity was not assessed. RAC notes that there were a large number of acceptable and supportive studies assessing germ cell mutagenicity following exposure to glyphosate.

Glyphosate is not electrophilic and is only metabolised to a limited degree as evidenced by the urinary excretion mainly of non-metabolised glyphosate. ADME studies show a wide tissue distribution of glyphosate following oral administration.

Mutagenicity and genotoxicity tests in bacteria and somatic cells

<u>*In vitro studies*</u>

The ability of glyphosate to cause mutations in bacteria was tested in 18 Ames tests with 17 performed in accordance with OECD TG 471 and 15 of these according to GLP. The majority of the tests were performed both with and without metabolic activation by an S9 pre-incubation step. All these tests and one bacterial DNA repair assay (Rec-assay, conducted according to US EPA FIFRA Guidelines and GLP) were negative, indicating that glyphosate is not mutagenic or genotoxic in bacterial systems. In addition to these 19 acceptable or supportive mutagenicity tests in bacteria, the DS included eight Ames tests, one Rec-assay and one *Escherichia coli* DNA repair assay (Pol A+/A-) with and without metabolic activation, that were not considered to be acceptable by the DS. No indications for mutagenicity were reported in these studies. All studies are included in table 49 of the CLH dossier. All five recommended strains were included in 14 studies. RAC noted that there is a concern that antimicrobial activity of glyphosate will prevent the growth of back-mutated Salmonella, thereby potentially producing false negative results in the Ames test. Cytotoxicity or reduced background growth of bacteria have been reported in a few of the Ames tests at high doses, but in most studies this was not the case. Furthermore, in a study by Shehata *et al*. (2013), *S. typhimurium* was reported to be relatively resistant to the growth inhibitory effect of glyphosate (minimal inhibitory concentration of 5 mg/mL). The conclusion that glyphosate is negative in bacterial mutagenicity tests is thus considered valid.

In mammalian cells glyphosate was tested in a range of *in vitro* studies for mutagenicity, clastogenicity and DNA damage or repair.

Five mammalian gene mutation tests were reported; three CHO/HGPRT gene mutation assays (CA 5.4.1/032, 1984; CA 5.4.1/040, 2021; and Report no. 31404, Volume 4 confidential information) and two mouse lymphoma tk locus assays (CA 5.4.1/030, 1998; CA 5.4.1/031, 1991). Glyphosate was negative both with and without S9 metabolic activation at concentrations up to 5 mg/mL (the current OECD TG 476/2016 requirement being 2 mg/mL) in the lymphoma assays and up to 25 mg/mL in the Chinese hamster ovary (CHO) cells.

One *in vitro* micronucleus (MN) test from 2021 performed according to OECD TG 487 (2016) was reported in human peripheral lymphocytes and was negative with and without S9 metabolic activation with concentrations up to 1268.25 µg/mL (the highest dose was equivalent to 10 mM) (CA 5.4.1/041, 2021). Some recent MN tests from the open literature were also assessed by the DS. Santovito *et al*., (2018, KCA 5.4/006) reported positive results in an MN test similar to OECD TG 487 in human lymphocytes with exposure to 0.0125 – 0.5 µg/mL glyphosate for 72h without metabolic activation. The DS considered this study to provide only supportive information due to methodological deviations. These included that no HCD was available, the purity of glyphosate was not characterised, the highest dose was not in line with the guidelines, the treatment started at 24h after stimulation instead of at 48h and the exposure duration was 48 h, thus exceeding the maximum exposure duration in OECD TG 487 of 1.5 cell cycles. Roustan *et al*. (2014, KCA 5.4/011) reported negative results (-S9) and positive results (+S9; ≥ 10 µg/mL) in CHO-K1 cells exposed to 5-100 µg/mL glyphosate (±S9) for 3h in a MN test (non-guideline, but similar to OECD TG 487). Further, no ROS formation was reported following exposure to glyphosate in this study. Limitations in the study included that no HCD was available, and no positive control was included. Kasuba *et al.* (2017, CKA 5.4/007) reported equivocal results with no clear dose-response for the induction of cytokinesis-block micronucleus (CBMN) in HepG2 cells with exposure to 0.5-3.5 µg/mL for 4 and 24h.

Two other *in vitro* MN tests from the public literature were included in the previous CLH dossier (CLH, 2016). The DS included only the information from the previous CLH dossier, and the RAC assessment of these studies have not changed from the RAC opinion from 2017. One of the studies was performed with human lymphocytes and was negative without S9 and positive in

samples with S9 activation at the highest concentration tested (580 µg/mL; Mladinic, 2009, ASB2012-11906 and ASB2012-11907). The second micronucleus test using a human buccal carcinoma cell line (TR146) exposed for a short period (20 minutes) to low glyphosate concentrations (10-20 µg/mL) was positive at the concentrations of 15 µg/mL and 20 µg/mL (Koller, 2012, KCA 5.4/013). At 20 µg/mL, increases in apoptosis and necrosis were reported, whereas the nuclear division index for cell integrity was reported to be unaltered by glyphosate exposure at these exposure levels. RAC notes that this cell line does not appear to be well characterised with respect to its performance in the *in vitro* MN test.

Glyphosate did not induce chromosomal aberrations (CA) in five of the seven *in vitro* studies presented in the CLH dossier, which were assessed in human peripheral lymphocytes or Chinese hamster lung cells, all ±S9 metabolic activation and at concentrations from 39-2000 µg/mL (CA 5.4.1/025, 1998; CA 5.4.1/026, 1996; CA 5.4.1/027, 995; CA 5.4.1/028, 1995; Mañas *et al.*, 2009, ASB2012-11892). The first four studies were reported as acceptable but with restrictions by the DS; however, it was noted that in the study CA 5.4.2/028 (1995) the top dose was not considered sufficiently high (1000 µg/mL). In the study by Mañas *et al.* (2009, ASB2012-11892) only 100 cells were scored per treatment, reducing the power of the experiment. Positive results were reported in two chromosome aberration tests using bovine and human lymphocytes exposed to low concentrations of glyphosate (Lioi 1998a, ASB2013-9836; Lioi 1998b; ASB2013-9837). These two studies were from the same laboratory and employed a non-standard exposure protocol. In the bovine study, cytotoxicity appeared (55% reduction of mitotic index) even at the lowest concentration level. The test using human lymphocytes reported increases in CA without any apparent reduction in mitotic index (Lioi, 1998b, ASB2013-9837).

From the public literature the DS included one study where the induction of CA was studied in human lymphocytes (Santovito *et al.*, 2018, KCA 5.4/006). The study reported positive results in a CA test similar to OECD TG 473 in human lymphocytes with exposure to low doses of glyphosate (0.0125 – 0.5 µg/mL) for 52h without metabolic activation. Limitations in the study included that no HCD was available, the highest dose was not in line with the guidelines, the treatment started at 24h after stimulation instead of 48h and the exposure duration was 28h (normally 3 - 6h), thus exceeding the maximum exposure duration in OECD TG 473 of 1.5 cell cycles.

One Cytogenetic Assay in CHO cells, ±S9, 62.5-1000 µg/mL (CA 5.4.1/029, 1989) was included by the DS. No indications of clastogenicity were observed, but the study was considered not acceptable due to major deviations in the study protocol.

Three Sister Chromatid Exchange (SCE) tests were reported (Lioi 1998a, ASB2013-9836; Lioi 1998b; ASB2013-9837; Bolognesi *et al.*, 1997, Z59299) and all found evidence of increased levels of SCEs in glyphosate exposed lymphocytes. RAC notes that the SCE was assessed in lymphocytes from only two female donors in the study by Bolognesi *et al.* (1997, Z59299), and in three donors in the studies by Lioi, *et al.* (Lioi 1998a, ASB2013-9836; Lioi 1998b; ASB2013-9837).

Two negative SCE studies were included by the DS, but these were considered not to be acceptable due to major deficiencies in the design of the studies (CA 5.4.1/038, 1993; CA 5.4.1/039, 1990).

Two negative Unscheduled DNS Synthesis (UDS) assays using primary hepatocytes was presented in the CLH dossier (CA 5.4.1/033, 1994; CA 5.4.1/034, 1984). The studies were considered to be not acceptable by the DS due to deviations from the OECD TG 482. RAC notes that the UDS assay is no longer a standard method and that the OECD TG 482 has been deleted.

Six *in vitro* Comet assays from the open literature were included by the DS and were considered as supportive by them. These assays had not been considered in the previous RAC Opinion on glyphosate from 2017. The studies used test procedures similar to OECD TG 489.

- In the first study, glyphosate did not induce cytotoxicity or genotoxicity in the Comet assay with exposure from 1-1000 µM ±S9 metabolic activation for 4h in human mononuclear white blood cells (Nagy *et al*., 2019, KCA 5.4/003).
- In the second study, glyphosate was positive in the Comet assay in endometrial cancer cells (HEC1A), negative in breast cancer (MCF7) cells and positive in the breast cancer (MDA-MB-231) cells with exposure to 500 and 1000 µg/mL for 4 hours. No information regarding metabolic activation was provided (De Almeida *et al*., 2018, KCA 5.4/004). One limitation was that at the highest concentration (800 - 1000 µg/mL) cytotoxicity was not assessed.
- In the third study, glyphosate was considered to be negative in the Comet assay in HepG2 cells exposed to low doses of glyphosate, 0.5 - 3.5 µg/mL for 4 and 24h without S9 metabolic activation (similar to OECD TG 489) (Kasuba *et al*., 2017, KCA 5.4/007). A decrease in tail intensity was reported after 4h, but not after 24h when compared to the control. A decrease in tail intensity might indicates DNA cross-links; however, according to OECD TG 489 this cannot be reliably detected with standard experimental conditions. Limitations in the study included lack of statistical significance, no reproducible effects between the 4h and 24h assessments, and the control values in the Comet assay were highly variable.
- In the fourth study, glyphosate was positive in the Comet assay in human peripheral blood mononuclear cells ≥ 0.5 mM (84.5 µg/mL) with exposure from 0.25 -10 mM (42.25 - 1690 µg/mL) glyphosate for 24h without metabolic activation (Kwiatkowska *et al*., 2017, KCA 5.4/008). However, after 120 minutes of recovery, significant repair of the DNA lesions was observed. The study included three donors; however, the description of the donors was limited. The study indicated that "healthy volunteers with no symptoms of infections" were used without any information regarding smoking medication or alcohol use included. No HCD was available.
- In the fifth study, glyphosate was positive in the Comet assay in human Burkitt's Lymphoma (Raji) cells exposed to 0.1 µM - 15 mM glyphosate for 24h without metabolic activation (Townsend *et al*., 2017, KCA 5.4/010). An increase in DNA damage was reported at ≥ 1 mM and glyphosate cytotoxicity was reported at ≥ 10 mM. The main deviations from OECD TG 489 were that the description of lysis conditions was incomplete, the number of scored cells too low, and no HCD was available.
- In the sixth study, glyphosate showed equivocal results in the Comet assay in a human-derived buccal epithelium carcinoma cell line (TR146 cell line) exposed to 10 - 2000 mg/L (Comet assay), and 10 - 200 mg/L (cytotoxicity assays) for 20 min without metabolic activation (Koller *et al*., 2012, KCA 5.4/013). An increase in tail intensity as compared to the controls at concentrations from 20 up to 2000 µg/mL was reported, with an increase between 20 and 40 µg/mL and no apparent further change in response up to 2000 µg/mL. Cytotoxicity was reported at ≥ 80 mg/L. Limitations in the study included that there was only a limited description of the test method, no positive control, no measurements of cytotoxicity between 200 and 2000 µg/mL and limited HCD.

RAC further notes that in a study by Suárez-Larios *et al*. (2017, CA 5.4/009) which was considered as supportive by the DS, the induction of DNA double strand breaks was studied in human peripheral blood lymphocytes exposed to 0.4 - 50 µM glyphosate for 1.5h without metabolic activation by immunofluorescence of phosphorylated (γ-H2AX) foci. No clear dose-response relationship was reported for the induction of double strand breaks.

Three *in vitro* Comet assays were included in the previous CLH dossier (2016) but were not submitted by the applicant. For these studies (Monroy *et al*., 2005; Mañas *et al*., 2009, ASB2012-11892; Mladinic *et al*., 2009b) the DS included the information provided in the CLH dossier (2016), and all three studies were reported as positive. Monroy *et al*. (2005) observed a genotoxic effect in human fibroblasts and fibrosarcoma cells from concentrations at or above 4 mM glyphosate.

In the study by Mañas *et al*. (2009, ASB2012-11892), DNA strand breaks were induced in Hep-2 cells of human epithelial origin at glyphosate concentrations between 507 and 1268 µg/mL (3 - 7.5 mM) with cytotoxicity at the highest dose level. Mladinic *et al*. (2009b) reported increases in tail intensity or tail length from 3.50 µg/mL glyphosate and above (the highest concentration being 580 µg/mL) in human lymphocytes both with and without S9 metabolic activation. These findings were seen together with an increased rate of early apoptotic and necrotic cells, an indication of cytotoxicity. In addition, Alvarez-Moya *et al*. (2014), considered as reliable with restrictions by the DS, tested glyphosate in human lymphocytes and reported an increase in tail length at all tested concentrations from 0.118 - 118 µg/mL (0.7 up to 700 µM), but the differences in DNA strand breaks between the concentrations were small and without a clear dose-response relationship. The study had several limitations, with untreated negative controls, only 50 cells or nuclei scored per slide, and it was unclear if cytotoxicity was assessed.

In summary, the *in vitro* data are not entirely consistent, but in a weight of evidence assessment the data indicate that glyphosate is not mutagenic. All Ames tests and mammalian gene mutation tests reported were negative. Five of seven chromosomal aberration tests were negative. Two tests from the same laboratory, both following an alternative protocol and therefore given less weight in the assessment, were positive. The micronucleus tests presented showed both positive and negative results, whereas the Comet assays indicated that glyphosate may induce DNA strand breaks or alkali labile sites in cultured cells.

The *in vitro* data have been corroborated by a range of *in vivo* genotoxicity and mutagenicity studies as described in the next section.

In vivo studies

Germ cell mutagenicity tests in rodents

Glyphosate was tested in three germ cell mutagenicity tests (rodent dominant lethal tests) (table 50 in the CLH dossier), one in Wistar rats (CA 5.4.1/001, 2014) with single doses up to 5000 mg/kg bw, one in CFY rats with an 8-week repeated exposure to 6.8, 20.5 or 70.4 mg/kg bw/d (CA 5.4.3/004, 2010), and one in CD-1 mice (CA 5.4.1/005, 2010) with doses up to 2000 mg/kg bw. The three studies were reported to be negative.

Non-human mammalian data

A considerable number of studies were available for the assessment of *in vivo* mutagenicity following exposure to glyphosate (table 50 of the CLH dossier). These consisted of bone marrow micronucleus and CA tests in rats or mice after oral or i.p. administration of glyphosate. Several toxicokinetic studies in rats are presented by the DS in section 2.6.1 of the CLH dossier and a summary of these studies is included in the section "RAC general comments" at the beginning of this opinion. It is noted that the MN studies were performed mainly in mice, with only one MN study in rats, and that no toxicokinetic studies are available in mice, limiting the assessment of the bioavailability of glyphosate in mice. The studies reported that approximately 20% of the glyphosate dose is absorbed in rats following oral administration with a rapid excretion of unchanged glyphosate via urine and faeces, which was complete within 24 - 48h. Further, the studies reported that glyphosate is widely distributed to body organs, including the bone marrow. It was noted that only low levels were measured in organs, with bone, bone marrow, carcass, liver, and kidney showing the highest levels in SD rats after a single oral dose of 30 mg/kg bw of glyphosate (CA 5.1.1./012, 1992). There was no evidence of accumulation of glyphosate, and the toxicokinetic and metabolism appears independent of sex, dose level, or repeated administration. There was also one study reported by the US EPA in rats with i.p. exposure to glyphosate. This study indicated that very low levels of glyphosate reached the bone marrow, and that rapid elimination from bone marrow occurs (MRID 00132685, 1983).

Negative results were reported in seven of the eight OECD test guideline compliant micronucleus tests in bone marrow cells, six studies in mice and one study in CD rats following oral exposure to glyphosate. The maximum doses for these studies were 2000 mg/kg bw or 5000 mg/kg bw given as single or double exposures, and all were performed according to OECD TG 474 and GLP. Four studies were considered acceptable by the DS, and three studies were acceptable with restrictions. In one of the MN studies, bone marrow exposure of glyphosate was confirmed by measurements of plasma levels of glyphosate 24h after exposure (Report no. 14613.402.078.14, 2015). This is in accordance with the OECD TG 474 "*Evidence of exposure of the bone marrow to a test substance may include a depression of the immature to mature erythrocyte ratio or measurement of the plasma or blood levels of the test substance*". One micronucleus test, (CA 5.4.2/010, 2017), demonstrated a statistically significant increase in the incidence of micronuclei in females at the high dose of 5000 mg/kg bw administered on two consecutive days (% MN-micronucleated polychromatic erythrocytes (MN-PCE): control 0.51; high dose 1.05), but not in males (%MN-PCE: control 0.69; high dose 0.89). RAC notes that the dose of 5000 mg/kg bw is above the guideline dose of max 2000 mg/kg bw. Furthermore, the control MN-PCE frequencies reported were higher than expected for this test. No increase in the percentage of micronuclei was observed at 50 or 500 mg/kg bw in the same study. No HCD for this study are mentioned in the CLH dossier. RAC agrees with the DS that biological significance of the weak positive result observed in the females at 5000 mg/kg bw is unclear. In addition, the DS included three not acceptable studies in mice with oral gavage, all negative for the induction of MN (CA 5.4.2/006, 2009; CA 5.4.2/011, 2007; CA 5.4.2/013, 1996). No effects on the PCE/normochromatic erythrocytes (NCE) ratio were reported in any of the oral micronucleus studies.

In addition to the oral studies, two mouse micronucleus tests in bone marrow cells were included by the DS following i.p. administration of glyphosate (from 188 to 600 mg/kg bw) and considered acceptable with restrictions by the DS. One of the studies, performed according to OECD TG 474 and GLP, showed no statistically significant increases in micronuclei (CA 5.4.2/008, 2008). The second study (CA 5.4.2/007, 2009) was considered to be negative, although reporting a statistically significant increase in %MN-PCEs at the high dose of 600 mg/kg bw (single dose) at the 24h sampling time. The level of MN-PCEs at the high dose (mean %MN-PCE in controls was 0.06 and 0.19 at high dose) was within the historical control range and was accompanied by a reduction in the PCE. RAC further notes that an increase in MN was not reported after the 48h sampling time. The DS also included one not acceptable study in mice with i.p. administration that was negative for the induction of MN (CA 5.4.2/004, 2010).

In addition to the regulatory guideline studies, four micronucleus tests from the open literature were assessed, three with positive results and one negative. Two of the studies (Ilyushina *et al*. (2018a, CA 5.4/002; 2018b, CA 5.4/005) were not included in the previous RAC opinion from 2017.

- In the first positive study (Mañas *et al*., 2009, ASB2012-11892), Balb-C mice (5 per dose, sex unclear) were included. A statistically significant increase in micronucleated erythrocytes (%MN cells in controls 0.38 and at high dose 1.3) was reported at 24h after the animals had received two i.p. doses of 200 mg/kg bw glyphosate, administered 24h apart. The two lower doses (2x50 or 2x100 mg/kg bw) were negative in this study. The study was reported by the DS to have some deviations from the OECD TG 474, the most problematic being that 1000 erythrocytes per animal were scored (instead of 4000 in the current test guideline, and 2000 at the time when the study was performed), and "erythrocytes" instead of immature or "polychromatic erythrocytes" were scored for micronuclei. RAC notes that it is unclear whether the authors have counted mature or immature erythrocytes as they did not specify this in the article. RAC also notes that counting as few as 1000 PCE (assuming PCE were counted) would give results which are

less reliable. The result from this study should be interpreted with care due to the deficiencies in reporting.

- In the second positive study (Bolognesi *et al*., 1997, Z59299), an increase (0.075% in control; 0.14% at 6h and 0.24% at 24h) in micronuclei in mouse bone marrow cells following two i.p. doses of 150 mg/kg bw on two consecutive days was reported. The study is limited in its methodological description. However, it reports four animals (instead of five) in each of the glyphosate exposure groups but counting of more cells (3000 vs 2000 NPCs per animal). The publication gives no reference to HCD.

- In the third study that was negative, four batches of glyphosate were tested for the induction of MN (purity of 95.7, 98.3, 95.1, or 95.8%) in mice exposed to 2000 mg/kg bw/d by gavage on two consecutive days (Ilyushina *et al*., 2018a, CA 5.4/002). The same authors performed a second study with three different batches of glyphosate (purity of 96.6%, 95.8% or 95.7%) at concentrations ranging from 500 to 2000 mg/kg bw/d (Ilyushina *et al*., 2018b, CA 5.4/005). Both studies were similar to OECD TG 474, were not conducted according to GLP, and were considered as supportive by the DS. In the second study, one of the three samples caused a statistically significant, dose-dependent increase in MN compared to the negative control. The authors postulated the presence of 0.13% formaldehyde in the respective batch (0.024% and 0.06% in the two other batches) as the cause for the positive result although they did not provide any data to support their hypothesis. The DS also noted that there is no evidence that formaldehyde induce systemic mutations (RAC opinion on formaldehyde, 2012).

Two chromosomal aberration tests are reported in the CLH dossier as supportive studies, both of which were negative. In the first study (CA 5.4.2/016, 1983), no CA were induced in rat bone marrow following i.p. exposure to 1000 mg/kg bw glyphosate with sampling 6, 12 and 24h after administration. In the second study in mice (CA 5.4.2/015, 1995), oral exposure to glyphosate at doses up to 2 x 5000 mg/kg bw did not induce an increase in CA.

RAC notes that low levels of glyphosate are distributed to the bone marrow, as well as the liver and kidney following oral, intravenous or i.p. exposure to glyphosate, and that glyphosate was rapidly excreted via the faeces and urine (see section "General RAC Comments" with more detailed information regarding the toxicokinetic of glyphosate).

Human data

The DS referred to the RAC opinion from 2017, which included the following statement: "*RAC finds that the interpretation of the human studies for the assessment of the genotoxicity of glyphosate is challenging due to the limited data available and confounding factors such as exposure also to other pesticides as well as uncertain exposure estimates. In addition, there is an issue with potential toxicity related to glyphosate based herbicide GBH co-formulants*".

However, some evidence was noted in two studies (described below) that investigated populations exposed to glyphosate based herbicide. These two studies were also discussed in the RAC opinion from 2017.

The DS did not assess the two studies. They were not provided by the applicant since they were > 10 years old. The DS had therefore copied the assessment from the RAC opinion from 2017 into the CLH dossier, noting that the study by Paz-y-Miño *et al*. (2007, ASB2012-11992) was considered as not reliable (Klimisch 3) due to higher application of glyphosate based herbicide (20 times higher) than recommended in the EU and that glyphosate based herbicide was combined with an adjuvant (Cosmoflux 411F, not used in EU) which can increase the biological action of the herbicide. The studies are presented in the RAR (section B.6.4.4.15) and suggest some evidence of genotoxicity in association with glyphosate based herbicide exposure.

Paz-y-Miño *et al*. (2007, ASB2012-11992) examined the consequences of aerial spraying with a glyphosate based herbicide added to a surfactant solution in people living in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the Comet assay two weeks to three months following intensive aerial spraying. The results showed a higher degree of DNA strand breaks in blood lymphocytes in the exposed group. However, individuals among the exposed group manifested clinical symptoms of toxicity after several exposures to aerial spraying which may by itself have an effect on generation of DNA single strand breaks. Further, RAC notes that the exposed groups also were co-exposed to glyphosate co-formulants.

Bolognesi and co-workers (2009, ASB2012-11570) reported on a binucleated MN biomonitoring study in subjects from five Colombian regions (N=274, approx. 60 subjects from each region), characterised by different exposures to glyphosate and other pesticides. One of the regions reported no use of pesticides including glyphosate. Blood samples were taken prior to spraying (indicative of baseline exposure to glyphosate before spraying), as well as five days and four months after spraying. The mean number of MN was greater in three regions (Nariño, Putumayo and Valle del Cauca) where the participants self-reported exposure, but the difference was not statistically significant, and in one of the regions (Valle der Cauca) only one participant reported contact with glyphosate (see table below). In the post-spray sample (four months), those who reported direct contact with the weedkiller spray showed a higher frequency of MN compared to those without glyphosate exposure. The increase in frequency of MN observed immediately after the glyphosate spraying was not consistent with the rates of application used in the regions and there was no association between self-reported direct contact with eradication sprays and frequency of MN. Further, the decrease in MN in the recovery period after glyphosate spraying (four-month sampling) was not consistent in the five regions since a statistically significant decrease in MN was only reported in one of the regions (Nariño). Bolognesi *et al*. (2009, ASB2012-11570) concluded that the data suggested that genotoxic damage associated with the glyphosate spraying as evidenced by the MN test was small and that no causal relationship between the increase in MN and glyphosate exposure could be established. Further, RAC notes that the exposed groups were also co-exposed to glyphosate co-formulants, as well as potentially being exposed to other pesticides.

Table: *results from the Bolognesi et al. (2009, ASB2012-11570) study*

Mean Frequency of Binucleated Cells with Micronuclei (BNMN) at the Second Sampling per 1000 Binucleated Lymphocytes and Self-Reported Exposures to the Glyphosate Spray in Three Areas Where Aerial Application Had Occurred

Route of exposure	Nariño (*n* = 55)		Putumayo (*n* = 53)		Valle del Cauca (*n* = 26)	
	n	Mean BNMN (SD)	*n*	Mean BNMN (SD)	*n*	Mean BNMN (SD)
No exposure	28	5.81 (1.85)	13	3.84 (1.30)	25	8.56 (2.90)
Spray in air	5	7.30 (0.57)	1	5.50 (0)		
Spray on skin	8	5.62 (1.60)	15	4.90 (1.87)	1	9.50 (0)
Entered sprayed field	14	6.06 (2.77)	24	4.87 (3.18)		
p Value (ANOVA)		0.472		0.612		0.760
Any exposure	27	6.16 (2.22)	40	4.90 (2.69)	1	9.50 (0)
p Value (no exposure vs. any exposure)		0.525		0.181		0.760

Note. The data comprise respondents in the second survey from which blood samples were obtained.

Other mammalian in vivo genotoxicity tests

Comet assay/alkaline elution assay

Two *in vivo* assays have been reported that measured the formation of DNA strand breaks and alkali labile sites in blood cells, liver, and kidney. An OECD test guideline (OECD TG 489) for the *in vivo* rodent Comet assay was used and the assay was validated by JaCVAM (Uno, 2015). The

information on these studies came from the previous CLH dossier (2016) since the studies were not submitted by the applicant.

In the study by Bolognesi *et al*. (1997, Z59299, considered to be not reliable in the previous CLH dossier, 2016), DNA strand breaks and alkali labile sites were measured by the alkaline elution assay in mouse liver and kidney cells 4h and 24h following single i.p. administration of glyphosate (300 mg/kg bw) to three male CD-1 mice. A transient induction of single strand breaks was detected in liver and kidney cells at the 4h time point but decreased to control values after 24h.

In a study by Mañas *et al*. (2013, KCA 5.4/012, considered to be a supportive study by the DS), induction of DNA strand breaks was examined in Balb C mouse peripheral blood cells and liver cells as measured by the Comet assay following exposure to doses of approximately 40 and 400 mg/kg bw/d glyphosate via drinking water for 14 days (6 mice/dose group). In this study an approximate doubling of the tail intensity measure was reported, with a dose-response relationship for liver cells; however, for blood cells no dose-related increased in the tail intensity was reported (see table below from the publication). However, RAC notes that the increase in the tail intensity was moderate in blood and liver cells. The methodological description in this publication is limited.

Table: *Comet assay in blood and liver cells of mice exposed to 0, 40 or 400 mg/kg bw/d glyphosate via drinking water for 14d (* p < 0.05, ** p <0.001, *** p < 0.0001, 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

These two studies suggest that glyphosate may induce increases in DNA strand breaks that are rapidly repaired following a single exposure. That glyphosate may induce increases in DNA strand breaks is supported by the *in vitro* Comet assays, but the data also appear to show that the increases in strand breaks reach a plateau with no further increase with increasing dose. The biological significance of a slight increase in DNA strand breaks as reported following exposure to glyphosate in the drinking water is uncertain.

Mechanistic studies - oxidative stress

The following studies in this section were not provided by the applicant (only Dai *et al*., 2016, KCA 5.6.1/023 was provided by the applicant) and the DS included summaries and evaluations of these studies from the previous RAR (2015). RAC has not changed their assessment of these studies and it is therefore the same as in the RAC opinion from 2017 (reproduced below).

"Measurements of DNA adduct levels and markers of oxidative stress may provide information on the potential genotoxic mode of action.

Bolognesi et al. (1997, Z59299) measured formation of the oxidative DNA lesion 8-hydroxy-2' – deoxyguanosine (8-OHdG) in liver and kidney from mice 8h and 24h following a single i.p. exposure to glyphosate (300 mg/kg bw). A statistically significant increase in 8-OHdG was reported in liver at 24h, but not after 8h and not in the kidney.

No increase in DNA adduct formation was detected by the ^{32}P-postlabelling method following i.p. exposure to glyphosate isopropyl ammonium salt to mice at a single dose of 130 or 270 mg/kg bw (Peluso et al., 1998, TOX1999-318).

Oxidative stress is characterized by an imbalance between generation of reactive oxygen species and anti-oxidant defence mechanisms, and can be measured as an increase in markers of oxidative stress such as malondialdehyde (MDA) e.g. by the thiobarbituric acid reactive substances (TBARS) assay.

In a study by Mladinic et al. (2009, ASB2012-11906) exposing isolated human whole blood samples to glyphosate in vitro, several markers of oxidative stress were examined. In this study an increase in plasma TBARS levels was demonstrated at the highest concentration of 580 µg/mL glyphosate. A modified version of the Comet assay was used with addition of the human 8-oxoguanine DNA glycosylase (hOgg1) that recognises the oxidised DNA lesion 8-OHdG. No consistent increases in Ogg1-sensitive DNA lesions were revealed over the concentration range tested.

A few studies (Mañas et al., 2009, ASB2012-11892 and 2013; Dai et al., 2016, KCA 5.6.1/023) have measured levels of lipid peroxidation by-products (MDA/TBARS) as putative makers of oxidative stress following in vivo exposures of mice or rats to glyphosate. Significant changes in MDA or TBARS were not reported in mouse tissues (liver, kidney, lung and heart) to single or repeated administrations of glyphosate, although some differences in activities of antioxidant enzymes were reported (Mañas et al., 2009, ASB2012-11892 and 2013, KCA 5.4/012, assigned a Klimisch score of 3 in the RAR). In a rat study (Dai et al., 2016, KCA 5.6.1/023) with doses up to 500 mg/kg bw/d for five weeks, no significant increases in testicular MDA levels or changes in anti-oxidant enzyme levels were reported. In addition, the IARC report and the RAR, 2015 both refer to a study in rats by Astiz et al. (2009, ASB2012-11549). This study measured effects on oxidative stress markers and oxidative defense systems in several tissues following repeated i.p. (10 mg/kg bw) glyphosate exposures three times a week for five weeks. TBARS concentrations in several tissues were increased (~doubled) in glyphosate exposed animals compared to the control animals, whereas plasma protein carbonyl levels were unaffected. In the RAR this study is given Klimisch code 3 due to deficiencies in reporting, low number of animals per group (4 rats/group), and i.p. route of administration. RAC notes that only the unexposed control data and not the vehicle control data are presented, and that the statistical evaluation seems to compare responses with the unexposed control data. The authors stated that they did not find any differences between data from the unexposed control group and the vehicle control group, but this is not shown."

More recently, several non-standard studies investigated the effects of glyphosate on oxidative stress and DNA damage or methylation in diverse *in vitro* cell systems was included by the DS (HepG2 cells (Kasuba *et al*., 2017, CA 5.4/007), human peripheral blood cells (Kwiatkowska *et al*., 2017, CA 5.4/008) and CHO-K1 cells (Roustan *et al*., 2014, CA 5.4/011). All three studies were considered as supportive information by the DS due to methodological shortcomings.

In addition, three new studies that measure ROS and oxidative stress have become available since the previous RAC opinion (2017). The first is a mechanistic study (non-guideline, non-GLP) by Gao *et al*. (2019) which was considered as reliable with restrictions by the DS. This study investigated the effects of glyphosate on renal proximal tubule cells *in vitro* and *in vivo* and is

further described under STOT RE and carcinogenicity. The *in vitro* part of the study showed that glyphosate (as a monoisopropylamine salt solution (40% w/w in water)) reduced cell viability, increased the incidence of apoptotic cells, and increased oxidative stress (MDA levels) in a concentration-related manner at 40 µM and above following exposure to 0, 20, 40, 50, 60, 70, 80, 90 or 100 µM. In the *in vivo* part of the study, kidney histopathology revealed exfoliation and increased apoptosis of renal tubular cells in male ICR mice (6 per group) treated with glyphosate at 400 mg/kg bw/d for 28 days. In addition, an oxidant/antioxidant imbalance and oxidative stress was observed. Based on this mechanistic study, the authors postulated that glyphosate could affect renal tubule epithelial cells via activation of the NMDA receptor signalling.

The second and third studies were two non-guideline, non-GLP studies by Liu and co-workers (Liu *et al.*, 2022a; 2022b), which were introduced during the presentation of key issues at RAC 60 by one civil society NGO and accepted by RAC for evaluation. These studies examined sperm quality and blood-testis barrier integrity in SD rats fed glyphosate in the diet at concentrations corresponding to 0, 2 or 50 mg/kg bw/d for 8 weeks (Liu *et al.*, 2022a) and for four months (Liu *et al.*, 2022b) (18 rats/group divided into three replicates/group, 6 rats/replicate). The reproductive toxicity data are presented under the section "Reproductive toxicity". An increase in an oxidative stress marker in the testis (MDA) was reported in the glyphosate treatment groups compared to controls. RAC notes that the results were not reported quantitatively in the studies, only in figures and images, thus limiting the assessment of the results, as well as the low number of animals used for some endpoints in the study. In an *in vitro* experiment (Liu *et al.*, 2022b), formation of ROS was observed in rat primary Sertoli cell cultures exposed to 10 µM glyphosate for 24h; however, RAC notes that no positive control was included in the study.

In addition, during the targeted consultation of glyphosate two studies assessing oxidative stress were included. In the first study by Eaton *et al.* (2022) urinary biomarkers of lipid oxidative stress in 347 urine samples collected between 16 - 20 weeks, and 24 - 28 weeks of gestation from pregnant women in the PROTECT birth cohort from Puerto Rico were studied. An increase in AMPA was associated with a higher level of urinary lipid oxidative stress biomarkers only during 24 - 28 weeks of gestation. Associations with glyphosate reflected similar trends, although findings were not as marked as for AMPA. For further information from the study, see the section describing developmental toxicity.

In the second study, Tang *et al.* (2020) studied the effects of glyphosate on the small intestine in male rats exposed to glyphosate by oral gavage to 0, 5, 50, or 500 mg/kg bw/d for 35 days. Indicators of oxidative stress were assessed, and they found a decreased activity of antioxidant enzymes (T-SOD, GSH, GSH-Px) and elevated MDA content in different segments of the small intestine. For further information from the study, see the section on STOT RE.

In general, it is considered that the investigated endpoints like oxidative stress, oxidative DNA damage and/or induction of proteins involved in DNA recombination do not directly measure effects on heritable mutations or events closely associated with chromosome mutations. Especially the stimulation of oxidative stress is not conclusively indicative for mutagenicity but may point to a possible mechanism of toxicity and induced cellular biological effects. Alterations in DNA methylation may not necessarily be indicative of genotoxicity, in addition to the mostly reversible nature of the epigenetic modifications. The toxicological relevance of the results reported by Kwiatkowska *et al.* (2017, CA 5.4/008) for classification for germ cell mutagenicity remains unclear. Overall, the *in vitro* and *in vivo* data suggest that glyphosate may induce oxidative stress. However, as glyphosate is considered non-mutagenic in the analyses of the large amount of standard mutagenicity tests, these additional mechanistic data are not considered to provide sufficiently conclusive evidence on genotoxicity. Therefore, they are given less weight in the assessment for classification of glyphosate for germ cell mutagenicity.

Comparison with the IARC evaluation

As the International Agency for Research on Cancer (IARC) previously concluded that glyphosate is genotoxic, a detailed comparison of the genotoxicity evaluation conducted by IARC and the DS is provided below.

The IARC Monograph (2015) is based on publicly available studies and does not consider data from unpublished reports, whereas the CLH dossier and the RAC opinions from 2017 and 2022 are based on both unpublished reports and publicly available studies resulting in a much broader data set for *in vivo* mammalian genotoxicity studies. In contrast to the RAC opinion, the IARC report also includes studies conducted in non-mammalian animal species. RAC further notes that there are no specific IARC criteria for genotoxicity to compare with the criteria in the CLP Regulation for germ cell mutagenicity.

IARC in their 2015 Monograph 112 concluded as follows:

"*There is strong evidence that glyphosate causes genotoxicity. The evidence base includes studies that gave largely positive results in human cells in vitro, in mammalian model systems in vivo and in vitro, and studies in other non-mammalian organisms. In-vivo studies in mammals gave generally positive results in the liver, with mixed results for the kidney and bone marrow. The end-points that have been evaluated in these studies comprise biomarkers of DNA adducts and various types of chromosomal damage. Tests in bacterial assays gave consistently negative results*."

There is a similar conclusion in the IARC Monograph and in the CLH dossier that glyphosate does not induce gene mutations in bacterial assays. In addition, one *in vitro* mammalian cell gene mutation study (Li and Long, 1988) was included in the IARC Monograph whereas three were included in the CLH dossier, but all were negative.

The *in vivo* bone marrow tests were given considerable weight in the IARC mutagenicity evaluation. One chromosomal aberration test (Li and Long, 1988) and three micronucleus tests (Rank, 1993, Z82234; Bolognesi *et al*., 1997, Z59299; Mañas *et al*., 2009, ASB2012-11892) were included in the IARC Monograph. All four studies were performed with i.p. administration of glyphosate; two were negative and two were positive. Accordingly, the IARC Monograph states that the bone marrow studies gave mixed results. All four studies were also assessed by RAC. RAC finds that deficiencies in design of the study by Mañas *et al*. (2009, ASB2012-11892) renders the biological relevance of the results uncertain, as commented above in the section describing "*In vivo* studies: Non-human mammalian data". Furthermore, RAC remarks that the micronucleus incidence in the high dose group in the study by Bolognesi *et al*. (1997, Z59299), is moderate and close to the control frequencies reported for other micronucleus tests. RAC has considered data from seven additional oral studies and three i.p. studies which were all negative and concludes that glyphosate is not mutagenic across the entire range of *in vivo* bone marrow mutagenicity tests.

Studies in exposed humans: The IARC Monograph concluded that there was positive evidence of DNA breakage in blood cells collected from two weeks to two months after spraying as determined by the Comet assay by Paz-y-Miño *et al*. (2007, ASB2012-11992). However, there was no induction of CA in blood cells from individuals in 10 communities who were sampled two years after the last aerial spraying with an herbicide mix containing glyphosate (Paz-y-Miño *et al*., 2011), nor an induction of MN in community residents after spraying compared to before aerial spraying with glyphosate-based formulations (Bolognesi *et al*., 2009, ASB2012-11570). However, IARC remarks that the increase in frequency of micronucleus formation observed immediately after spraying was not consistent with the rates of application used in the regions, and there was no association between self-reported direct contact with pesticide sprays and frequency of binucleated cells with micronuclei.

RAC notes that the results from the human genotoxicity studies are equivocal and that their overall interpretation is challenging due to the time between spraying and blood sampling (from two weeks to two months), uncertain exposure estimates and the combined exposures to glyphosate and co-formulants and also to other pesticides. RAC concludes that the data available are not sufficient to conclude that glyphosate is the factor likely to explain the association between glyphosate-based herbicide and higher incidences of micronuclei in the studies where this has been observed.

<u>Supporting evidence/indicator tests</u>

The IARC Monograph 112 (2015) stated that "*In-vivo studies in mammals gave generally positive results in the liver, with mixed results for the kidney …*".

RAC notes that two studies (Bolognesi *et al*., 1997, Z59299; Mañas *et al*., 2013, KCA 5.4/012) report induction of DNA single strand breaks in liver following either a single i.p. or a repeated oral exposure.

Mechanistic studies – oxidative stress

IARC reported that "*there is strong evidence that glyphosate, glyphosate-based formulations, and aminomethylphosphonic acid can act to induce oxidative stress based on studies in experimental animals, and in studies in humans in vitro. This mechanism has been challenged experimentally by administering antioxidants, which abrogated the effects of glyphosate on oxidative stress. Studies in aquatic species provide additional evidence for glyphosate-induced oxidative stress.*" On page 69 it states that: "*Specifically, it was found that glyphosate induces production of free radicals and oxidative stress in mouse and rat tissues through alteration of antioxidant enzyme activity, depletion of glutathione, and increases in lipid peroxidation. Increases in biomarkers of oxidative stress upon exposure to glyphosate in vivo have been observed in blood plasma (Astiz et al., 2009b), liver (Bolognesi et al., 1997, Z59299; Astiz et al., 2009b), skin (George et al., 2010), kidney (Bolognesi et al., 1997, Z59299; Astiz et al., 2009b), and brain (Astiz et al., 2009b).*"

RAC has evaluated the rodent studies with regard to markers of oxidative stress, with the exception of the study by George *et al*. (2010) where dermal exposure to a glyphosate containing formulation showed reduced expression of the antioxidant enzyme (SOD) in skin. RAC considers the study by Astiz *et al*. (2009) to be of uncertain reliability due to deficiencies in the reporting. In addition to the studies evaluated in the IARC Monograph, RAC has included data from the *in vivo* studies by Mañas *et al*. (2009, ASB2012-11892; 2013, KCA 5.4/012), Dai *et al*. (2016), and the recent studies by Gao *et al*. (2019), Liu *et al*. (2022a; 2022b), Tang *et al*. (2020) and Eaton *et al*. (2022). RAC considers the data from the studies to be equivocal due to deficiencies in reporting and concludes that there is some evidence that glyphosate may induce oxidative stress in certain cells and tissues.

Comparison with the CLP criteria

The database available for evaluation of germ cell mutagenicity is extensive and includes studies covering bacterial and mammalian cell *in vitro* mutagenicity assays as well as *in vivo* mammalian mutagenicity assays and some human data. The database includes studies of sufficient reliability and relevance to allow a robust evaluation following the requirements of the CLP Regulation. Mutagenicity data related to exposures to AMPA and glyphosate-based herbicide are not considered in this analysis by RAC as the purpose is to provide a harmonised classification of glyphosate itself, the exception being the inclusion of human biomonitoring data. Genotoxicity data from non-mammalian species are not included in the assessment, because the relevance of the findings to humans of such studies conducted using non-standard protocols is less clear than

in the many studies available which were conducted using standard protocols and standard animal models, and for the majority of the studies conducted under GLP.

<u>Category 1A</u>

According to the CLP criteria, classification of a substance as a germ cell mutagen in Category 1A is based on positive evidence from epidemiological studies showing transmission of DNA damage to progeny in humans following exposure (heritable mutations in germ cells).

A limited number of biomonitoring studies have examined markers of possible genotoxicity in blood cells from humans exposed occupationally or from the general population in regions with high use of glyphosate. Some of these studies showed an apparent positive relationship between exposure to glyphosate and the levels of the markers being studied. However, all these studies were compromised by the lack of clear information about exposure to glyphosate itself and glyphosate-based formulations, and the extent to which other substances or lifestyle factors could have contributed to the findings. In some cases, the low numbers of subjects involved was also a factor. Although not completely negative, these studies do not provide sufficiently robust evidence of glyphosate germ cell mutagenicity to justify classification for this hazard class.

The classification of glyphosate as Muta. 1A is not justified.

<u>Category 1B</u>

According to the CLP criteria, classification of a germ cell mutagen in Category 1B is largely based on positive result(s) from *in vivo* heritable germ cell mutagenicity tests in mammals; or from *in vivo* somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations in germ cells.

There was no evidence for mutagenic activity in germ cells of mice or rats at oral doses up to 2000 and 5000 mg/kg bw, respectively, in the dominant lethal tests presented. However, given that glyphosate has a wide distribution in the body, exposure of germ cells is considered likely; therefore, results from the somatic mutagenicity studies are relevant also for the evaluation of germ cell mutagenicity. However, RAC notes that from toxicokinetic studies very limited amounts of glyphosate are reported in the testis and ovary.

The bacterial mutation assays and mammalian cell gene mutation tests gave consistently negative results. Furthermore, a total of seven oral and seven i.p. bone marrow micronucleus tests and two chromosomal aberration tests in rodents were reported. All oral tests and three of the i.p. tests were conducted according to OECD TG 474 or 475 and performed according to GLP. The majority of these bone marrow test were negative, but two were positive. One was considered to have deficiencies making the interpretation uncertain and was hence given less weight in the overall assessment. The other presented a statistically significant increase that may well have been within the anticipated control level. Thus, the evidence from these two positive studies does not override the overall conclusion from the numerous other *in vivo* mutagenicity studies, that glyphosate does not induce somatic cell mutations. However, RAC notes the concern relating to the presence of glyphosate in sufficient amounts to induce MN in bone marrow.

The mammalian *in vivo* database is considered sufficient; however, RAC notes the absence of a reliable *in vivo* Comet assay according to OECD TG 489 in relevant tissues as well as a TGR somatic and germ cell gene mutation assay conducted according to OECD TG 488 (the Guideline was recently updated for the analysis of mutations in germ cells). In an overall weight of evidence assessment of the available data, it is considered that glyphosate does not induce germ cell mutation and a classification as Muta. 1B is not warranted.

Classification in Category 2 is largely based on positive evidence obtained from somatic cell mutagenicity tests in mammals or other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays.

Glyphosate is only metabolised to a very limited degree and is not a DNA reactive substance. Bacterial and mammalian gene mutation and clastogenicity assays were all negative. Thus, the genotoxicity observed for glyphosate in some studies is likely to be caused by indirect mechanisms. Glyphosate appears to induce transient DNA strand breaks as observed in the *in vitro* and *in vivo* Comet assays or by using the alkaline elution assay; however, no reliable *in vivo* Comet assays were included in the CLH dossier in relevant target organs e.g., liver and kidney or a TGR somatic and germ cell gene mutation assay. There is also some evidence that glyphosate may induce oxidative stress in certain cells and tissues with the potential to induce oxidative DNA-lesions that may lead to mutations if not repaired. However, the gene mutation assays were all negative and bone marrow mutagenicity was considered negative in a weight of evidence assessment of the available oral and i.p. micronucleus assays. Noting the absence of a Comet assay conducted according to OECD TG 489 in relevant tissues as well as a TGR somatic and germ cell gene mutation assay conducted according to OECD TG 488, the biological importance of such DNA lesions in relation to mutagenicity is equivocal. Taking all data into account and based on the overall negative responses in the existing gene mutation and oral mutagenicity tests, RAC concludes that **no classification of glyphosate for germ cell mutagenicity is warranted**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

In the CLH dossier, studies using mice and rats as well as epidemiological studies addressing the effects of exposure to glyphosate in humans were assessed by the DS. These studies and their findings are discussed in detail below.

The main statistical methods used in the animal studies were the Fisher's exact test for pairwise comparisons and the Cochran-Armitage trend test, and in this opinion these two methods are referred to unless stated otherwise. In their detailed assessment of findings, the DS repeated both the pairwise and trend test statistical calculations for the findings from relevant studies (eight studies in rats and five studies in mice; for details, see below). In addition, for one study in mice (CA 5.5/016, 2001), a Peto-analysis was performed for the induction of malignant lymphomas. RAC also notes that the DS included available historical control data (HCD) for the selected tumour types, in order to make a comparison with the natural background levels.

Background information on the assessment of glyphosate includes several recent reviews (EFSA, 2015; JMPR, 2016; FSC Japan, 2016; PMRA Canada, 2017; US EPA, 2019). In these assessments it was concluded that glyphosate is not a carcinogen. RAC concluded in its opinion in 2017 (RAC, 2017) that no classification for carcinogenicity is warranted. However, in 2015, a review by the IARC concluded that the evidence for the carcinogenicity of glyphosate was limited in humans but sufficient in experimental animals (rats and mice). IARC further concluded that glyphosate was probably carcinogenic in humans.

Due to the IARC conclusion, experts have investigated why there are different conclusions from different investigating bodies (Crump *et al*., 2020, B.6.5.18.1; Portier *et al*., 2020, B.6.5.18.2).

Crump *et al.* (2020, B.6.5.18.1) pointed out that the animal carcinogenicity data on glyphosate are extensive (≥ 15 long term rodent oral bioassays of glyphosate identified by US EPA (2016), EFSA (2016) and IARC (2015). Each bioassay was conducted in both sexes, with each sex potentially having 40-60 unique tumour types, resulting in over 1000 potential statistical tests, which could result in many statistically significant (p ≤ 0.05) tumours by chance alone – approximately 5%. Crump *et al.* (2020, B.6.5.18.1) further assessed the probability of false positives using a modification of the permutation approach of Farrar and Crump (1988 and 1990). The analysis made by Crump *et al.* (2020, B.6.5.18.1) showed that statistically significant effects on tumour incidences should be carefully evaluated for biological relevance as chance findings may occur.

Portier *et al*. (2020, B.6.5.18.2) also provided an additional revised statistical evaluation and trend test analyses of relevant tumour types reported in the carcinogenicity studies but did not take into account the chance effect due to multiple testing as pointed out by Crump *et al.* (2020, B.6.5.18.1). Furthermore, as indicated in the OECD Guidance document 116, statistical significance is only part of the interpretation of the biological importance of a particular finding. In the CLH dossier, as well as in the current RAC assessment, the tumour types showing statistically significant trends in the analysis by Portier *et al*. (2020, B.6.5.18.2) were taken into consideration in the assessment of cancer types. One of the differences between the study by Portier *et al*. (2020, B.6.5.18.2) and the analysis by the DS was that Portier used 1-sided testing with a significance level of 0.05, whereas in the original study reports and the DS analysis 2-sided testing was applied with a significance level of 0.05 (which is equivalent to 1-sided testing using a significance level of 0.025).

Rats

The DS noted that they were aware of eight unpublished long-term feeding studies with the technical active ingredient in rats (summarised in table 53 of the CLH dossier) of which six were performed in compliance with OECD TG 453. The DS concluded that the remaining two studies (CA 5.5/003, 1997; CA 5.5/011, 1981) were "flawed by serious deficiencies", but since tumour data from one of these studies (CA 5.5/011, 1981) were subject to debate with regard to some of the observed tumour types, the DS also took this study into consideration together with the six guideline-compliant studies.

The DS noted that the main carcinogenicity findings in rats comprised an increase in islet cell tumours of the pancreas (CA 5.5/019, 1990; CA 5.5/011, 1981), increases in liver tumours and in C-cell adenoma of the thyroid (CA 5.5/010, 1990), and an increase in interstitial cell tumours of the testis (CA 5.5/011, 1981). The DS also noted that the following tumours were not discussed in the previous RAC opinion from 2017.

- Pituitary adenoma in rats. Portier *et al*. (2020, B.6.5.18.2) highlighted a statistically significant trend in the incidence of pituitary adenomas in male and female rats in the study CA 5.5/001 (2009).
- Skin basal cell tumours and skin keratoacanthomas in rats. Portier *et al*. (2020, B.6.5.18.2) highlighted a statistically significant trend in these types of tumours in male rats.

The DS assessed each of these findings in detail also taking note of the assessment by Portier *et al*. (2020, B.6.5.18.2). In the remaining four GLP compliant studies in rats conducted according to OECD test guidelines, no increases in tumour incidences were seen. In the case of the pancreatic tumours, the DS noted that at the low dose in males (but not at the two higher doses or in females), when compared pair-wise to the concurrent controls, a re-evaluation of the data confirmed a statistically significant increase in adenomas in the study CA 5.5/010 (1990) (dose range 89 - 940 mg/kg bw/d) and in the study CA 5.5/011 (1981) (dose range 3 - 31.5 mg/kg bw/d), with an increase in adenomas and carcinomas combined in the latter. However, this study

was considered to be unacceptable by the DS due to the low doses used compared to the other carcinogenicity studies as well as the low quality of the study report. Furthermore, the DS noted a statistically significant positive trend for carcinomas in male animals in the CA 5.5/011 (1981) study, which had not been previously reported. This was seen in a single affected male at the high dose, but in none of the other animals. There were no incidences of pancreatic tumours in the females. No dose-response relationship was observed and there was no indication of progression to malignant neoplasia in either study. The DS also noted that an increased incidence of pancreatic tumours was not reproducible in other (#5) more recent and OECD TG compliant studies, in which the incidences of pancreatic cancer in untreated control animals sometimes resembled the incidences reported in these two studies.

The incidence of liver tumours in the study CA 5.5/010 (1990) was assessed in the RAC opinion from 2017. In addition, the DS included the assessment of the incidence of liver tumours in the study CA 5.5/002 (2001). From the study CA 5.5/010 (1990), a re-evaluation was performed by the previous DS (Germany; CLH, 2016) of glyphosate using trend- and pair-wise tests. A statistically significant trend was confirmed for the adenomas, but no positive trend was observed for the adenoma and carcinoma combined. The DS also noted that a dose-response relationship "*was hardly to be seen*" and although absolute and relative liver weights were increased in high dose males in the study, there were no pre-neoplastic findings that might progress to liver tumours. In the study CA 5.5/002 (2001), hepatocellular adenoma was observed in five out of 64 male rats (7.8%) in the high dose group (1214 mg/kg bw/d) compared to 0 in the control group and two adenomas in the low dose group. The study reported that the incidence at the top dose was not statistically significant using the pairwise test. The difference was, however, statistically significant using the Peto-test for trend, but there was no clear dose-response relationship and no progression to carcinoma was reported. It was noted that the incidence of hepatitis at the top dose was above the HCD mean (11.8%) but within HCD range (0 - 30%; HCD based on five studies from the same laboratory and in the same strain between 1998 and 2003). As the background incidence of hepatitis is highly variable and as the incidence is within HCD range, the relationship to treatment was considered by the DS to be doubtful. The other four carcinogenicity studies in rats (two studies with Wistar rats, two studies with Sprague-Dawley rats) did not show an effect on hepatocellular adenomas, and in addition, no incidences of liver tumours were reported in female rats. The DS concluded that the observed increase in hepatocellular adenomas was considered incidental and not related to treatment.

Increases in the incidence of C-cell adenoma in female rats was seen in the study CA 5.5/010 (1990) which were negative using a pairwise comparison, but weakly positive in the trend test (p=0.0435). In the absence of such a finding in any of the other rat studies, this increase in C-cell tumours was not considered by the DS to be biologically significant.

An increased incidence in interstitial testicular tumours was observed in CA 5.5/011 (1981). Although there was no clear dose-response relationship, at the top dose the difference relative to the control was statistically significant (p < 0.05). The DS noted that in the original study report it was argued that the absence of this tumour type in the control group was unusual and that the high dose incidence was "only marginally above the historical control range" and no increase in testicular tumours was observed in any other long-term study with glyphosate in rats, despite much higher doses having been administered.

The publication by Portier *et al*. (2020, B.6.5.18.2) highlighted a statistically significant trend in in the incidence of pituitary adenoma in male and female rats in the study CA 5.5/001 (2009). However, the DS had doubts about the trend test used that reported tumour incidences as incidence/number of animals investigated in low and mid dose group and not incidence/per total number of animals/groups. Furthermore, no increased incidences of pituitary adenomas were

reported in the other rat carcinogenicity studies, and the DS concluded that glyphosate did not induce pituitary adenomas.

In the publication by Portier *et al*. (2020, B.6.5.18.2) skin basal cell tumours and skin keratoacanthomas in male rats were highlighted. These tumours were not extensively discussed in the previous RAC opinion from 2017.

Skin basal cell tumours: Portier *et al*. (2020, B.6.5.18.2) reported a positive trend for skin basal cell tumours in male Sprague-Dawley from the study CA 5.5/004 (1997), and the trend was confirmed by an external statistician upon request by the DS (2-sided p=0.001 for the extended Mantel-Haenszel test (stratified Cochran-Armitage trend)). The study reported an incidence of three benign adenomas and one malignant carcinoma in the high dose group (1127 mg/kg bw/d) with the absence of tumours in the control, low and mid dose groups. No skin basal cell tumours were reported in the female rats. The DS noted that the apparent increase in skin basal cell tumours was only observed in one study in males and not in the three other studies with Sprague-Dawley rats nor in the three studies with Wistar rats. Only limited HCD for this tumour were available (only two studies); therefore, the DS considered it difficult to put the findings into perspective. The DS considered the finding as being of equivocal relevance and not sufficient for classification.

Skin keratoacanthomas: Portier *et al*. (2020, B.6.5.18.2) highlighted the increased incidences of skin keratoacanthomas in male rats in four studies (CA 5.5/004, 1997; CA 5.5/007-009, 1993; CA 5.5/010, 1990 and CA 5.5/001, 2009) with no findings in two studies (CA 5.5/002, 2001; CA 5.5/005, 1996). The DS considered that the increased incidence of skin keratoacanthomas were reported at very high dose levels, which slightly exceeded the maximum recommend dose levels of 1000 mg/kg bw/d according to the OECD test guideline. The only exception was the study CA 5.5/010 (1990), where a dose-response relationship was reported, but which was not linear (1.7%, 5.0%, 6.7% and 8.5% in the dose groups exposed to 0, 89, 362, 940 mg glyphosate/kg bw/d). However, in one study in Wistar rats (CA 5.5/002, 2001) at the same high lose level (1214 mg/kg bw/d), no increase in skin keratoacanthomas was reported. No non-neoplastic precursor effects were observed, and no malignant squamous cell carcinomas were reported in the studies. The DS noted that although the incidences exceeded the background incidence (for which limited information was available for most of the studies), no statistically significant differences were observed (either by pairwise comparison or by trend analysis; 2-sided testing). However, a statistically significant increase was found by Portier *et al*. (2020, B.6.5.18.2) when a 1-sided trend test (Cochran-Armitage) was used in the study CA 5.5/004 (1997) (p=0.029); CA 5.5/007-009 (1993) (p=0.047) and CA 5.5/010 (1990) (p=0.042). In a weight of evidence assessment, the DS considered that the apparent increase in skin keratoacanthomas was not of sufficient relevance for classification.

The DS also noted another 2-year carcinogenicity study in rats (Pavkov and Wyand, 1987). In this study, glyphosate was administered as a trimesium salt at dose levels of 0, 4.4/5.4, 21.1/27.0 and 41.1/55.7 mg/kg bw/d (males/females). This study was not mentioned in the previous EU review on glyphosate; however, a study summary was provided by the Applicant. According to the summary provided, there were no treatment-related increases in tumour incidences in the study. The Applicant informed that glyphosate trimesium has not been manufactured since 2003 and not sold since 2004. Furthermore, the Applicant noted that glyphosate trimesium has been regulated as a separate active ingredient to glyphosate itself.

Mice

The DS summarised and assessed five OECD TG 451 compliant long-term studies in mice (table 53 in the CLH dossier). In two of the studies (CA 5.5/018-019, 1997; CA 5.5/023, 1983), high doses greater than 4000 mg/kg bw/d had been administered and the DS noted these doses were

above the OECD limit dose of 1000 mg/kg bw/d. In addition to the five OECD TG 451 compliant studies, the DS also noted the existence of two long-term studies in mice, which "*did not comply with current standards*" (CA 5.5/022, 1988; CA 5.5/024, 1982 original report, revised 1992) and were considered as unacceptable. In these two studies, no increase in any tumour type had been reported, but the high dose was considered much too low for a meaningful evaluation of carcinogenicity (300 ppm). Furthermore, limited parameters were investigated, the number of animals/dose group was too low, and the study reports had limited quality. In addition, the DS noted an 18-month feeding study in male and female CD-1 mice (Anonymous, 1999), which was mentioned in the Joint FAO/WHO Meeting on Pesticide Residues (JMPR, 2016) evaluation on glyphosate. . According to the JMPR analysis, an increased incidence in malignant lymphomas was reported in female mice. However, the study report was not available to the DS, and it was not possible to assess the reliability and the incidences of tumours in the study. The DS also noted another 2-year carcinogenicity study in CD-1 mice in a US EPA assessment (Pavkov and Wyand, 1986), in which glyphosate was administered as a trimesium salt (purity 56.2%) at dose levels of 0, 11.7/16, 118/159 or 991/1341 mg/kg bw/d (males/females). This study was not mentioned in the previous EU review on glyphosate; however, a study summary was provided by the Applicant. According to the summary provided, there were no treatment-related increases in tumour incidences in the study. As noted above, the Applicant informed that glyphosate trimesium has not been manufactured since 2003 and not sold since 2004. Furthermore, the Applicant noted that glyphosate trimesium has been regulated as a separate active ingredient to glyphosate acid itself.

In the studies assessed, there was evidence of increased incidences of three types of tumours (malignant lymphoma, renal tumours, and haemangiosarcoma; all in males), which were addressed in detail in the CLH dossier.

Malignant lymphoma was reported in four studies with CD-1 mice, as well as in a study using Swiss mice. In addition, a sixth study was available (JMPR, 1999) in CD-1 mice where only female mice were assessed. These tumours were also highlighted by Portier *et al*. (2020, B.6.5.18.2), and a new statistically analysis was conducted by the Applicant and the DS as well as updated historical control data set when compared to the previous EU evaluation. The DS noted that the statistical significance of the suspected increase in malignant lymphoma in the various studies was very much dependent on the statistical method used for analysing the data. In the studies CA 5.5/012-015 (2009) and CA 5.5/018-019 (1997), the findings were statistically significant when the trend test was applied (1- and 2-sided), but not when a pairwise comparison was performed (but statistically significant with a 1-sided pairwise test, Portier *et al*., 2020, B.6.5.18.2). The increased incidence in the study CA 5.5/016 (2001) was not confirmed either by the trend test (1- and 2-sided) or by a 2-sided pairwise test but only when using a 1-sided pairwise test and 1-sided Peto-analysis. In the mouse study from 1999, reported in the 2016 JMPR evaluation, no HCD were available, and incidences of malignant lymphoma were reported only in female mice. The DS noted that an extremely high dose was used (8690 mg/kg bw/d). The incidence of malignant lymphoma in female mice was 2% in the low dose group, 8% in the intermediate dose group, 12% in the high dose groups vs 6% in the control group. The results were not statistically significant with a Fisher exact test (pair-wise comparison, JMPR, 2016) as well as in a trend test (1-sided, Portier *et al*., 2020, B.6.5.18.2, $p=0.05$ and 2-sided $p > 0.05$, JMPR, 2016).

The DS concluded that based on an inconsistent dose-response relationship in the individual studies, and a highly variable spontaneous tumour incidence as suggested by the HCD, also noting that malignant lymphomas are among the most common spontaneously occurring neoplasms in the mouse, it was not likely that glyphosate induced malignant lymphoma in mice. The DS also noted that a possible role of oncogenic viruses should not be ignored in the study in Swiss mice (CA 5.5/016, 2001). The DS also questioned the human relevance of an effect which

was only seen at high doses. As regards the Anonymous (1999) study reported by JMPR (2016) the DS noted the high doses used in the study (8-9-fold higher than recommended in the OECD TG) and considered that the slight increase in malignant lymphoma has low biological significance. In summary, the current assessment of the induction of malignant lymphoma did not add any new information that would change the outcome of the previous RAC evaluation in 2017.

Renal tumours were reported in three studies with CD-1 mice and the study using Swiss mice. A re-evaluation of the histopathological findings from the CA 5.5/023 (1983) study in CD1 mice by a Pathology Working Group was conducted (EPA, 1986). These tumours were highlighted by Portier *et al*. (2020, B.6.5.18.2), and a new statistically analysis was available as well as updated HCD. The DS concluded that the renal tumours in male mice were not likely to be treatment related, primarily because the incidences of the findings were not statistically significant in comparison with concurrent controls, but also because the incidences at the highest doses were similar to those in controls in other studies, the findings were within the historical control ranges, were not reported in female mice, there were no pre-neoplastic lesions in treated animals and there was no plausible mechanism.

Evidence for development of haemangiosarcoma was seen in male CD-1 mice at the highest dose in two studies (CA 5.5/020-021, 1993; CA 5.5/018-019, 1997). These tumours were also highlighted by Portier *et al*. (2020, B.6.5.18.2). The incidences were not statistically significant in comparison with the concurrent controls by a pairwise comparison but were statistically significant using a trend test. The DS noted that the findings were within the historical control range. The DS concluded that the incidence of haemangiosarcomas reported in two studies in CD-1 mice is unlikely to be treatment related since no effects were reported in two other studies with CD-1 mice, nor in Swiss mice and were within the historical control range.

In addition, mesenteric lymph node haemangioma was reported in one study in female Swiss mice (CA 5.5/016, 2001) and was not discussed in the previous EU evaluation, but was highlighted by Portier *et al*. (2020, B.6.5.18.2). Since this tumour type was not reported in females in the other mouse studies, the DS considered the effects as incidental and not treatment related.

Humans

The DS summarised a number of epidemiological studies investigating the relation between glyphosate exposure and cancer and included both case-control and cohort studies (table 54 in the CLH dossier). In addition, reviews, statistical re-analyses, systematic reviews, and meta-analyses of already published data were available and were also assessed by the DS. The DS noted that a general concern with the epidemiological studies was that no accurate glyphosate exposure assessment was available, and therefore all exposure assessments were based on questionnaires instead of measurements, e.g., biomonitoring data.

One of the main studies included by the DS is the data collected in the Agricultural Health Study (AHS) cohort from the USA, also noting further publications arising from the AHS cohort, which has been updated since the RAC evaluation of glyphosate in 2017. In this update, data were prospectively collected from more than 57000 farmers (users of crop protection products) in the USA. In addition to the previous assessment of glyphosate the DS included the following recent main studies:

Pahwa *et al*. (2019) combined the data from two case-control studies and reported a weak association with the occurrence of non-Hodgkin's Lymphoma in a subgroup working > two days/year with glyphosate and the occurrence and diffuse large B-cell lymphoma (odds ratios of 1.7, 95% CI: 1.0 - 2.9 and 2.1, 95% CI: 1.1 - 4.3, respectively). However, the DS noted that this only concerned a very small research population of n=30 and n=14 cases, respectively, and

the DS considered it uncertain how representative the results were for the entire population, and the results from the study should be interpreted accordingly.

Andreotti *et al*. (2018) showed that, based on the data from the AHS cohort, no overall association between exposure to glyphosate-based herbicides and cancer was reported. However, a weak association can be seen for persons with a relatively high exposure (third tertile) and acute myeloid leukaemia and non-Hodgkin's Lymphoma after a 20-year lag time (time between exposure and tumour development). These data also concern a very small research population of n=15 and n=8 cases, respectively, and therefore the DS considered these findings to be of questionable value. However, the DS noted the finding of a possible association with acute myeloid leukaemia should be looked at carefully in future updates on the AHS data. The DS, however, noted that a high number of cancer sites were analysed so there was the possibility of statistical findings by chance and that acute myeloid leukaemia was not observed in any of the other epidemiological studies with glyphosate.

The DS noted that as already reported in the previous evaluation (CLH, 2016; RAC, 2017) some of the case-control studies reported slightly increased Odds Ratios (OR) for certain tumours. However, most of these studies had limitations such as a lack of adjustment for confounders including other pesticide exposure or lifestyle factors, were based on a very low number of exposed cases and/or had a high proportion of proxy responders. Adjusting for confounding factors such as exposure to other pesticides was shown to lower the ORs in most of the studies where such an exercise was conducted. Proxy responders were also found to lead to higher ORs than self-responders (e.g., Lee *et al*., 2005).

Further, the DS noted that non-Hodgkin's Lymphoma is not a specific disease, but a broad spectrum of disorders more correctly referred to as lymphocytic lymphomas, each with possible different aetiologies. They are all classified as not being Hodgkin's Lymphoma, and the terminology has changed over the years - some lymphomas are described differently today compared to previously. This complicates the evaluation of the studies.

The DS concluded that a causal relationship to cancer following exposure to glyphosate-based herbicides is not proven. Although a few of the available epidemiological cohort and case-control studies showed weak statistically significant associations between exposure to glyphosate-based herbicides and findings of cancer (non-Hodgkin's Lymphoma or a subtype and acute myeloid leukaemia), chance, bias and confounding factors could not be ruled out.

Mechanistic studies from public literature

Five studies were included by the DS assessing possible modes of action following exposure to glyphosate. One *in vivo* study in wild-type (WT) mice and Vk*MYC (multiple myeloma animal model) mouse assessing the effect of exposure to glyphosate and multiple myeloma, and four *in vitro* studies assessing epigenetic effects, autophagic effects and lipid accumulation following exposure to glyphosate. The studies are presented in table 55 in the CLH dossier.

From the *in vitro* studies it was noted that glyphosate did not affect lipid accumulation in 3T3-L1 adipocytes (Biserni *et al*., 2019) and did not induce an autophagic effect in human A549 cells (Hao *et al*., 2019).

Glyphosate reduced global DNA methylation level and altered DNA methylation in promotor regions of P21 and P53, with no changes in P16, BCL2 and CCND1 in human peripheral blood mononuclear cells. The gene expression was decreased for P16 and P53 and increased for BC12, CCND1 and P12; however, no clear dose-responses were reported for these changes and the changes in DNA methylation profile were minimally correlated with gene expression (Wozniak *et al*., 2020).

Duforstel *et al*. (2019) assessed the DNA methylation pattern in non-neoplastic MCF10A cells and reported that low doses of glyphosate (10^{-11} M) induced DNA hypomethylation with TET3 overexpression. The study concluded that glyphosate may promote development of mammary tumours but is not considered as oncogenic. However, since only one low concentration of glyphosate was tested, no dose-response relationship could be determined.

In the *in vivo* study by Wang *et al*. (2019) WT mice and Vk*MYC mouse were exposed to glyphosate, 1 g/L in drinking water for 72 weeks (corresponding to 90 mg/kg bw/d). More severe effects were reported in the Vk*MYC mouse compared to the WT mouse on survival, spleen weight, Immunoglobulin G (IgG) levels and haematology. In WT mouse, glyphosate induced benign monoclonal gammopathy (mouse equivalent to monoclonal gammopathy of undetermined significance in humans) and promoted multiple myeloma progression in Vk*MYC mouse. The study was judged to have several limitations, included low number of animals in each group, only one dose tested, and low exposure to glyphosate compared to the chronic guideline studies in mice. Further, the effects reported on haematological parameters were reported at higher dose levels in the chronic mice studies. The DS considered that the study did not have any direct impact on the overall assessment of glyphosate.

<u>Conclusions of the DS</u>

The DS concluded that based on the epidemiological data as well as on data from long-term studies in rats and mice, taking a weight of evidence approach, no classification for carcinogenicity is warranted for glyphosate according to the CLP criteria.

Comments received during consultation

Comments no. 21 - 53 submitted during consultation were related to carcinogenicity.

Twenty-two comments supported the DS proposal for no classification for carcinogenicity. These comments were provided by Company manufacturers, Company downstream users, National/International civil society NGOs, Individuals, MCSAs, National Authorities and Industry or Trade Organisations. Eight comments supported a classification for carcinogenicity. These comments were from Industry or Trade Organisations, International/National NGOs, National Authorities, Academic institutions, and individuals.

Four comments were from MSCAs, with three supporting the position of the DS for no classification for carcinogenicity. One MSCA commented that the impact of all new epidemiological data for a possible classification of glyphosate (no classification vs Carc. 2; H351), should be included in the assessment and discussed.

Comments supporting classification generally focused on the fact that IARC (2015) had classified glyphosate as a probable carcinogen Group 2A (equivalent to Cat. 1B in the CLP Regulation) based on limited evidence in humans, strong evidence from animal studies and strong evidence for genotoxicity. Other comments noted that there are several publications on oxidative stress, both on glyphosate and the metabolite AMPA. Furthermore, commenters noted that there was an incorrect assessment of the carcinogenicity from animal studies by the DS with special attention to there being no systematic scientific review of the 23 chronic carcinogenicity/toxicity studies available for glyphosate, the statistical method used (1-sided vs 2-sided tests and pairwise vs trend test), incorrect use of HCD, the evaluation of the consistency of response of the same tumour in multiple studies and excluding findings at the top-dose if they were exceeding the limit dose. Furthermore, they stated that an appropriate weight of evidence assessment was not included.

Other commenters noted that the recent epidemiological reviews and meta-analysis from the open literature not included in the CLH dossier by the DS should be assessed for a possible classification of glyphosate as Carc. 2/1B, (Weisenburger, 2021; Kabat *et al*., 2021; Leon *et al*., 2019; Zhang *et al*., 2019 and a new epidemiological case-control study by Meloni *et al*., 2021). Other comments noted that the AHS cohort only included a short follow up period (median lifetime years of glyphosate use 8.5 years with a median follow up of 18 years), that 37% of the participants did not complete the follow up questionnaire, and therefore less weight should be put on this cohort study compared to the case-control studies. One comment criticised the study by Crump *et al.* (2020, B.6.5.18.1) noting that the study underestimates the case-control studies, pointing out that these studies were performed by experienced epidemiologists using widely accepted study designs and methods, were published in peer-reviewed journals, and were acceptable for review and consideration.

The DS responded that the assessment of the available data for carcinogenicity has been done according to the many EFSA guidance documents on the assessment of active substances under the Plant Protection Products (PPP) Regulation, OECD test guidelines and the CLP guidance. Furthermore, the DS noted that the assessment of the animal carcinogenicity studies included statistical analysis based on values reported in the original study reports, the statistical re-assessment of the data given in the previous CLH dossier (2016) and the DS' own statistical analysis and included both 1-sided and 2-sided testing. However, the DS noted that statistical significance is not the only criterion to decide on classification for carcinogenicity. The opinion of the DS was that the interpretation of the tumour incidences observed among the carcinogenicity studies in rats and mice should be made based on a weight of evidence assessment, as required by the CLP Regulation, which balances statistical analysis and biological plausibility. As regards the assessment of oxidative stress, the DS responded that the evaluation from RAC in 2017 was taken into consideration as well as new data from the public literature. As regards the comments that the DS excluded findings at the top-dose since these exceeded the limit dose, the DS noted that the OECD TG 453 states that "*a limit of 1000 mg/kg bw/d may apply except when human exposure indicates the need for a higher dose level to be used*". Thus, for glyphosate a top dose of 1000 mg/kg bw/d is considered appropriate as the exposure to humans is far below this level.

The DS noted that the latest AHS publication is an interim report as the AHS is still ongoing and that no clear association was seen between exposure to glyphosate and cancer in the AHS data that were reported up to now. The DS also noted that the AHS cohort is the only prospective cohort study available. As regards the Crump *et al.* (2020, B.6.5.18.1) study, the DS noted that the purpose of the analysis by Crump *et al*. was to evaluate the evidence for recall bias in the overall pattern of results in the five case-control studies and two cohort studies. These studies comprise the main part of the glyphosate non-Hodgkin's Lymphoma literature, and that that all submitted epidemiological studies have been evaluated according to EFSA recommendations for their reliability. As regards the recent epidemiological studies and meta-analysis (Meloni *et al*., 2021; Andreotti *et al*., 2018; Zhang *et al*., 2019; Leon *et al*., 2019; Kabat *et al*., 2021), which are more recent than the 2017 RAC evaluation, in their response the DS supported including these studies in a weight of evidence analysis based on animal and human data and considering statistical as well as biological significance of the results in the comparison against the CLP criteria.

Assessment and comparison with the classification criteria

Non-human data

No new carcinogenicity bioassays in rats and mice were included by the DS compared to the RAC opinion from 2017. Nine rat and seven mouse carcinogenicity bioassays were included in the CLH dossier (table 53 in the CLH dossier). Two of the rat and two of the mouse carcinogenicity bioassays were considered to be unacceptable by the DS and while not rejecting them, RAC

considers that these studies have less weight in the assessment of carcinogenicity (CA 5.5/003, 1997; CA 5.5/011, 1981; CA 5.5/022, 1988; CA 5.5/024, 1982, revised 1992). The limitations included, amongst others, unknown purity of the substance tested, several parameters in OECD TG 453 were not included in the analysis, low doses, few dose levels tested and low number of animals/dose.

Therefore, seven rat and five mice carcinogenicity bioassays form the major basis of the current RAC evaluation of carcinogenicity in animals, as was the case in RAC's 2017 assessment.

RAC assessed the data from the original full study reports (robust study summaries of which are included in the RAR). In the original study reports, mostly pairwise comparisons had been made, whereas in the IARC Monograph (2015), trend tests were the preferred statistical tool. The DS included the statistical significance of the observed tumour incidences by the use of both pairwise comparisons by the Fisher's exact test, and trend analysis by the Cochran-Armitage trend test (1-sided and 2-sided trend tests), and their respective p-values when available are included in the assessment by RAC in this opinion. Due to the many cancer bioassays performed for glyphosate, RAC notes that for the assessment of carcinogenicity the evaluation of biological relevance of an increased tumour incidence is critical and is given more weight compared to statistically significance in the weight of evidence assessment.

RAC notes that when using trend tests, significant trends are in some cases related to smaller increases in tumours only reported in the high dose group with no or low incidences in the control group. In these cases, provided the findings were not significant in pairwise testing, the strength of the evidence was considered to be weak.

RAC further notes that some changes have been made by the DS relating to the historical control data for the tumour types assessed in rats and mice compared to that included in the RAC opinion from 2017; however, these changes did not have any impact on the overall assessment of the carcinogenicity studies in rats and mice by the DS nor by RAC.

Rat combined chronic toxicity/carcinogenicity studies

Study selection - rat bioassays

Nine long-term studies were available to RAC for the assessment of carcinogenicity in rats following exposure to glyphosate, with six of the studies performed according to OECD TG 453 (Combined Chronic Toxicity/Carcinogenicity Studies). One study, regarded by the DS to be unacceptable due to significant reporting deficiencies and insufficient dose levels (CA 5.5/011, 1981), was included in the carcinogenicity assessment by the DS and by RAC due to the occurrence of pancreatic and testicular tumours. This study used low doses, thus not satisfying the guideline requirements. A study using adequate dose levels has subsequently been performed (CA 5.5/010, 1990). In addition, the DS found the negative study CA 5.5/003 (1997) not suitable for evaluation of classification since several parameters were not investigated in the study, moreover it had an unusually low background incidence of tumours so no clear conclusion can be drawn from this study. RAC agrees with this assessment. Finally, one of the carcinogenicity studies was designed with an exposure to glyphosate for one year (CA 5.5/006, 1996). The exposure to glyphosate was 0, 2000, 8000 or 20000 ppm corresponding to 0, 141, 560 or 1409 mg/kg bw/d in males and 0, 167, 671 or 1664 mg/kg bw/d in females. No treatment related tumours were reported; however, RAC notes that a 1-year study is not adequate for the assessment of carcinogenicity.

RAC notes that the IARC Monograph included the studies CA 5.5/002 (2001), CA 5.5/007-009 (1993), CA 5.5/010 (1990) and CA 5.5/011 (1981), but not the studies CA 5.5/001 (2009), CA 5.5/004 (1997) and CA 5.5/005 (1996).

According to the DS, no evidence of carcinogenicity was observed in the long-term rat studies after an evaluation of all data. IARC stated that there were no increases in tumour incidences in the glyphosate treated groups in the studies CA 5.5/007-009 (1993) and CA 5.5/002 (2001). However, IARC pointed out a significant increase in the incidence of pancreatic islet cell adenoma in males in two Sprague-Dawley rat studies (CA 5.5/011, 1981; CA 5.5/010, 1990) and that the latter study also showed a significant positive trend in the incidences of hepatocellular adenoma in males and of thyroid C-cell adenoma in females. RAC has evaluated the neoplasias of the rat pancreas, liver, thyroid, testes interstitial cell tumours, pituitary adenomas, skin basal cell tumours and skin keratoacanthomas based on data provided in the CLH dossier and the RAR.

The suggestion of increased incidences in tumours of the pancreas, liver, thyroid, testes interstitial cell tumours, pituitary adenomas, skin basal cell tumours and skin keratoacanthomas are mainly based on findings in the study CA 5.5/010 (1990), with support for pancreatic tumours also from the study CA 5.5/011 (1981) and skin tumours from the studies CA 5.5/001 (2009), CA 5.5/004 (1997) and CA 5.5/007-009 (1993), and testis tumours from CA 5.5/011 (1981). There were no significant effects on body weight noted in males of any dose group in the study CA 5.5/010 (1990). In high dose females, body weights were statistically significantly reduced from week 7 to approximately the 20th month.

Pancreatic islet cell tumours

In the table below, the incidences of pancreatic islet cell tumours in male rats in all seven studies are shown.

Table: *Incidences of pancreatic islet cell adenomas and carcinomas combined in male rats*

Study [strain]	Control	Low dose incidence (%) [mg kg bw/d]	Mid dose incidence (%) [mg kg bw/d]	Second incidence (%) [mg kg bw/d]	High dose incidence (%) [mg kg bw/d]	Response Fisher's exact test
CA 5.5/001, 2009 [Wistar]	4 / 51 (7.8%)	1 / 51 (2.0%) [86]	2 / 51 (3.9%) [285]	-	1 /51 (2.0%) [1077]	No significant increase
CA 5.5/002, 2001 [Wistar]	1 / 53 (1.9%)	2 / 53 [121]	0 / 53 [361]	-	1 / 52 (1214]	No significant increase
CA 5.5/004, 1997 [Sprague-Dawley]	4 / 50 (8.0%)	1 / 50 (2.0%) [104]	2* / 50 (4.0%) [354]	-	1 / 50 (2.0%) [1127]	No significant increase
CA 5.5/005, 1996 [Wistar]	3 / 48 (6.3%)	0 / 30 (0.0%) [6.3]	0 / 32 (0.0%) [59]	-	1 / 49 (2.0%) (595]	No significant increase
CA 5.5/007-009, 1993 [Sprague-Dawley]	7 / 50 (14.0%)	1 / 24 (4.2%) [10]	2 /17 (11.8%) [100]	2 / 21 (9.5%) [300]	1 / 49 [1000]	No significant increase
CA 5.5/010, 1990 [Sprague-Dawley]	2* / 43 (4.7%)	8 / 45 (17.8%) [89]	5 / 49 (10.2%) [362]		7 / 48 (14.6%) [940]	Significant increase in adenoma at low dose vs control
CA 5.5/011, 1981 [Sprague-Dawley]	0 / 50 (0.0%)	5 / 49 (10.2%) [3]	2 / 50 (4.0%) [10.3]	-	3* / 50 (6%) [31.5]	Significant increase in adenoma at low dose vs control

*including one carcinoma

Two of the nine studies show an increase in pancreatic adenomas (CA 5.5/010, 1990; CA 5.5/011, 1981).

In the study CA 5.5/010 (1990), an increase in pancreatic islet cell adenomas was reported, but the increase did not reach statistical significance when using the Cochran-Armitage trend test. The pairwise Fisher's exact test was only positive for the low dose group compared to controls.

Further, there was no progression to malignancy in the exposed groups since the only carcinoma was reported in the control group. In this study, no pancreatic islet cell carcinomas were reported in females and the adenoma incidences (5/60, 1/60, 4/60, and 0/59, at the control, low, mid and high doses, respectively) did not show an increase in exposed groups versus controls. There were no dose-related increases in pancreatic hyperplasia in male or female rats suggesting that the adenomas were spontaneous and not treatment related.

According to the RAR, the incidences of adenomas in males (17.8%, 10.2% and 14.6% at the low, mid and high doses, respectively) were outside the historical control range (1.8 – 8.5%, mean 5.3%) for this laboratory.

In the study CA 5.5/011 (1981), no clear dose-related increase in pancreatic islet cell adenomas and carcinomas was reported. However, when using the pairwise Fisher's exact test a statistically significant increase in adenoma was reported in the low dose group, but not in the two higher dose-groups. When using the Cochran-Armitage trend test a statistically significant increase was found for carcinomas (p=0.046), but not for adenomas. Due to the low doses used in this study and the toxicokinetic studies showing low absorption of glyphosate (approx. 30%), the statistically significant increased incidences of adenomas in the low dose group is considered to rather be due to the unusually low (0) incidences in the control group. RAC notes the low quality of the study report.

The elevated incidences of pancreatic adenomas observed in glyphosate-exposed groups in the two studies discussed above were only observed in males and did not show a dose-response relationship. Furthermore, they were not supported by findings in the additional five long-term guideline studies in rats (table above) in which no increase in pancreatic islet cell tumours were reported in response to glyphosate. In four of these studies, the incidences were higher in the control groups than in the glyphosate exposed groups. The findings were not considered to be strain-dependent as the two other studies in Sprague-Dawley rats did not show any increases in pancreatic islet cell tumours.

Liver tumours

Table: *Liver adenomas and carcinomas in male rats in the CA 5.5/010 (1990) and CA 5.5/002 (2001) studies*

Dose (mg/kg bw/d)	Male rats	Liver adenoma (p-values)	Liver adenoma + carcinoma (p-values)
CA 5.5/10			
0	60	3	6
89	60	2 (1.000)	4 (0.739)
362	60	3 (1.000)	4 (0.732)
940	60	8 (0.162)	10 (0.392)
Cochran-Armitage Trend test (p-value)		0.0171	0.0752
CA 5.5/002			
0	64*	0	0 (no significant increase)
121	64*	2	2 (no significant increase)
361	64*	0	0 (no significant increase)
1214	64*	5	5 (no significant increase)
Cochran-Armitage Trend test (p-value)		< 0.05	

p-values in brackets when using Fisher's exact test. Statistically significance, p-values < 0.05.
*12 of the 64 animals sacrificed after 1 year.

A positive trend for liver adenomas was reported in study CA 5.5/010 (1990) in male Wistar rats (table above). The increase in adenomas was statistically significant when using the Cochran-Armitage trend test, but not in the pairwise testing against controls (Fisher's exact test). There was no progression to malignancy in the exposed groups as the incidence of liver carcinomas was slightly higher in controls than in the glyphosate treated groups. No statistically significant increase was reported for liver adenomas and carcinomas combined. At the interim sacrifice, relative liver weights were slightly, but statistically significantly increased in high dose males whereas absolute and relative liver weight was increased in high dose males at the end of the study. No pre-neoplastic liver lesions were reported in the CLH dossier or the RAR.

The hepatocellular adenoma incidences in the glyphosate treated animals (5, 3.3, 5 and 13.3% in the control, low, mid and high dose group, respectively) were within the historical control range from the test facility and the same strain (6.7 - 18.3%) but slightly higher than the mean of 11.1%.

The CA 5.5/002 (2001) study was not included in the RAC assessment for liver tumours in the opinion from 2017; however, Portier *et al*. (2020, B.6.5.18.2) highlighted the increase in liver tumours in this study and it is included in the current assessment. Liver adenomas were reported in male Sprague-Dawley rats (table above) with only one incidence in the mid dose female rats. Liver adenoma in males was observed in five out of 64 animals (7.8%) compared to no incidences in controls. The incidence of liver adenoma in the high dose group was not statistically significant using the Fisher's Exact test; however, the difference was statistically significant using a trend test.

It is noted that, although a statistical trend is observed, no clear dose-response was reported (0, 2, 0 and 5 adenomas in the control, low, mid and high dose group, respectively). It is also noted that there was no progression to carcinomas. The incidence of adenomas in high dose males of 7.8% was slightly outside the HCD (range 0 - 5.8%, mean 1.5%; HCD from 5 studies between 1998 and 2003). At the interim sacrifice, the liver weight was statistically significantly decreased in the high dose male rats. It was noted that an increased incidence in hepatitis was reported in high dose males. The reported incidences were 8/64, 6/64, 9/64, 13/64 in the control, low, mid and high dose group, respectively. The incidence of hepatitis in the high dose group was above the HCD mean (11.8%) but within the HCD range (0 - 30%; HCD based on 5 studies from the same laboratory and in the same strain performed between 1998 and 2003). RAC notes that as the background incidence of hepatitis is highly variable and the incidence is within the HCD range, the relationship to treatment is not clear.

No significant increases in glyphosate-related liver tumours were reported in the other long-term studies in rats.

Thyroid C-cell tumours

Table: *Thyroid C-cell adenomas and carcinomas in CA 5.5/010 (1990) study*

Dose (mg/kg bw/d)	Female rats Adenomas (%); Carcinomas (%)	Fisher's exact test	Male rats Adenomas (%); Carcinomas (%)
0	2/60 (3.3%); 0/60 (0%)		2/60 (3.3%); 0/54 (0%)
89	2/60 (3.3%); 0/60 (0%)	NS	4/58 (6.9%); 2/58 (3.4%)
362	6/60 (10.0%); 1/60 (1.7%)	NS	8/58 (13.8%); 0/58 (0%)
940	6/60 (10.0%); 0/55 (0%)	NS	7/60 (11.7%); 1/60 (1.7%)

Dose (mg/kg bw/d)	Female rats Adenomas (%); Carcinomas (%)	Fisher's exact test	Male rats Adenomas (%); Carcinomas (%)
Cochran-Armitage trend test (p-value)	p=0.0435 (adenomas)		Non-significant

Thyroid C-cell tumours were discussed in the RAC opinion from 2017. An increase in the incidence of thyroid C-cell adenomas was reported for both sexes in the CA 5.5/010 (1990) study and a significant trend was found for female rats using the Cochran-Armitage test with a p-value of 0.0435. No statistical significance was found when using pairwise comparison (Fisher's exact test with Bonferroni inequality). For males, the increased incidences of adenomas or combined adenomas/carcinomas were not statistically significant. No progression from adenoma to carcinoma was indicated in this study. Further, no effects on non-neoplastic precursors were reported, and the thyroid was not reported to be a target organ following glyphosate exposure in the repeated dose toxicity studies. Only in the 1-year study in dogs, a higher thyroid weight accompanied by C-cell hyperplasia was noted in males.

The thyroid C-cell adenoma incidences in the high dose glyphosate treated animals were slightly higher than the historical control range (3.3 - 8.5%, mean 6.7% in females and 5.0 - 8.6%, mean 6.8% in males from three studies between 1986 and 1989). No increase in thyroid C-cell adenomas was reported in the other long-term studies in rats. In these other studies, there were no increases in pre-neoplastic histological lesions and no thyroid weight change was noted in response to glyphosate exposure.

Interstitial cell tumours in the testis

Table: *Interstitial cell tumours in testis in CA 5.5/011 (1981) study*

Dose (mg/kg bw/d)	Interstitial cell tumours in the testis
0	0/50
3.0	3/50
10.3	1/50
31.5	6/50 (p < 0.05, Fisher exact test)

An increased incidence in interstitial cell tumours in the testis was reported in the CA 5.5/011 (1981) study, noting that this study was considered as unacceptable by the DS and testicular tumours were not discussed by RAC in the opinion from 2017. The dose levels used in the study were very low compared to the other rat carcinogenicity studies, and the quality of the study report was poor. However, since this tumour type was only reported in this study, RAC considers it relevant to discuss this in the current opinion. In the high dose group, statistical significance was reached in a pairwise comparison. However, no clear dose-response relationship was reported, and it was noted that this is a common tumour in aging rats. The incidence in the high dose group was in the CLH dossier described to be slightly outside HCD; however, there was a lack of relevant HCD provided by the applicant. Only one study from 1980 - 1982 was provided, with an incidence of 5% (4/80 rats); however, RAC notes that since the HCD are from only one study this has limited value. An increased incidence of interstitial cell tumours in the testis was not reported in any of the other carcinogenicity studies in rats and mice, so overall RAC considers that glyphosate does not induce interstitial cell tumours in the testis of rats as was also concluded in the RAC opinion from 2017.

Pituitary adenoma

Table: *Pituitary adenoma in CA 5.5/001 (2009) study*

Dose males (mg/kg bw/d)	Pituitary adenoma males incidence	Dose females (mg/kg bw/d)	Pituitary adenoma females incidence
0	16/51	0	24/51
85.5	11/18	104.5	23/28
285.2	10/18	348.6	16/25
1077.4	20/51	1381.9	32/51

Note: In the low and mid dose groups histopathological examination was only performed on animals that died pre-terminally and that were moribund sacrificed.

Pituitary adenoma was not assessed in the RAC opinion from 2017, but was highlighted in the Portier *et al*. (2020, B.6.5.18.2) paper. In the study CA 5.5/001 (2009), no increase in any tumours were reported by the DS in the CLH dossier and the RAR. However, Portier *et al*. (2020, B.6.5.18.2) highlighted an increased incidence of pituitary adenoma in the CA 5.5/001 (2009) study and described a statistically significant trend in male and female rats (1-sided p=0.045 and p=0.014, respectively), with no increase in pituitary carcinomas (one carcinoma reported in the male control group, and one in the female low dose group). However, RAC as well as the DS note that the trend test including the low and mid dose is not considered appropriate since the histopathological examination was only performed on animals that died pre-terminally and that were sacrificed moribund. Further, tumour incidences were reported as incidences/number of animals investigated and not as incidences/total number of animals/dose group. RAC further notes that pituitary adenomas are a common tumour in rats when taking into account the incidences of this tumour in the other rat carcinogenicity studies (table 2.6.5.1-5a in the CLH dossier). Overall, RAC considers that glyphosate does not induce pituitary adenomas in rats.

Skin tumours

Skin tumours were not assessed in the RAC opinion from 2017. However, Portier *et al*. (2020, B.6.5.18.2) highlighted a positive trend for skin tumours in the rat carcinogenicity bioassays including skin basal cell tumours and skin keratoacanthomas and this was also discussed by the DS.

Skin basal tumours

Table: *Incidences of skin basal cell tumours in male rats*

Study, year	Control	Low dose	Mid dose	Second mid dose	High dose	Trend test Cochran-Armitage
CA 5.5/001, 2009	1/51	0/51 86 mg/kg bw/d	0/51 285 mg/kg bw/d		0/51 1077 mg/kg bw/d	Not analysed
CA 5.5/002, 2001	1/64	0/64 121 mg/kg bw/d	2/64[#] 361 mg/kg bw/d		1/63 1214 mg/kg bw/d	Not analysed
CA 5.5/004, 1997	0/76	0/75 104 mg/kg bw/d	0/80 354 mg/kg bw/d		**4/78[#a]** 1127 mg/kg bw/d	p=0.001
CA 5.5/005, 1996	0/50	0/30 6 mg/kg bw/d	0/32 59 mg/kg bw/d		0/50 595 mg/kg bw/d	Not analysed
CA 5.5/00-009, 1993	1/50	0/25 10 mg/kg bw/d	0/19 100 mg/kg bw/d	0/21 300 mg/kg bw/d	0/50 1000 mg/kg bw/d	Not analysed
CA 5.5/010, 1990	0/59	0/60 89 mg/kg bw/d	0/60 362 mg/kg bw/d		1/59 940 mg/kg bw/d	Not analysed

In the CA 5.5/004 (1997) study, a statistically significant trend for the induction of skin basal cell tumours were found in male rats (0/76, 0/75, 0/80, 4/78 in the control, 104, 354 and 1127 mg/kg bw/d group, respectively; p=0.001, for adenoma and carcinoma combined). At the high dose, three adenomas and one carcinoma were reported. An increased trend in skin basal cell tumours was only reported in the male rats in the CA 5.5/004 (1997) study and not in the three other studies in Sprague Dawley rats, nor in the three studies in Wistar rats. Limited HCD was provided, only from two studies (1995 - 2000) with 0% for both adenomas and carcinomas in male rats. RAC notes that in the CA 5.5/004 (1997) study, a statistically significantly increased incidence of follicular hyperkeratosis was reported with an incidence of 29.5% (23/78) in top dose males and 8% in top dose females (6/78) compared to 9.2% and 0% in controls for males and females, respectively, which might indicate a precursor effect.

RAC notes that in the study CA 5.5/002 (2001), one adenoma and one carcinoma was reported in the mid dose, one adenoma in control group and one adenoma in the high dose group (1/64, 0/64, 2/64 and 1/63 in the control, 121, 361 and 1214 mg/kg bw/d group, respectively). Historical control data was provided from five studies (1998 - 2003) with 0% for both adenomas and carcinomas in male rats. Since no dose-response relationship was evident for the induction of skin basal tumours, and no skin basal tumours were reported in the other five carcinogenicity studies in male and female rats, RAC considers this to be a chance finding.

Overall, RAC considers that the increased trend of skin basal tumours only reported in the CA 5.5/004 (1997) study and not in the five other carcinogenicity studies in rats, nor in female rats with limited HCD is of equivocal relevance. Further, no clear effects on the skin were reported following systemic exposure to glyphosate in the repeated dose toxicity studies in animals; therefore, RAC considers that the reported incidences of skin basal cell tumours are not sufficient for classification.

Skin keratoacanthomas

Table: _Incidences of skin keratoacanthomas in male rats_

Study [strain]	Control (%)	Low dose (%) [dose]	Mid dose (%) [dose]	Second mid dose (%) [dose]	High dose (%) [dose]	HCD	Trend test Cochran-Armitage (p-values)
CA 5.5/004, 1997 [Sprague-Dawley]	4/76 (5.3%)	3/75 (4%) [104]	0/80 (0%) (354)		7/78 (9.0%) [1127]	2 studies, 4% and 8%	**1-sided: 0.029** 2-sided 0.21
CA 5.5/007-009, 1993 [Sprague-Dawley]*	1/50 (2%)	2/25 (8%) [10]	0/19 (0%) [100]	0/21 (0%) [300]	5/50 (10%) [1000]	13 studies range 0 - 6.1%, mean 0.7%	**1-sided: 0.047** 2-sided 0.07
CA 5.5/010, 1990 [Sprague-Dawley]	1/59 (1.7%)	3/60 (5.0%) [89]	4/60 (6.7%) [362]		5/59 (8.5%) [940]	3 studies: 1/6, 1/5 and 0/2**	**1-sided: 0.042** 2-sided 0.15
CA 5.5/001, 2009 [Wistar]	2/51 (3.9%)	3/51 (5.9%) [86]	0/51 (0%) [285]		6/51 (11.8%) [1077]	No HCD	**1-sided: 0.03** 2-sided Not available
CA 5.5/002, 2001 [Wistar]	1.6%	0% [121]	1.6% [361]		1.6% [1214]	No HCD	1-sided: 0.387 2-sided Not available

| CA 5.5/005, 1996 [Wistar] | 0% | 0% [6] | 0% [59] | | 0% [595] | No HCD | Statistical analysis not performed |

* Only animals that died during the study or that were killed in extremis were investigated in low, mid and second mid dose groups (25, 19 or 21 animals/group, respectively). Therefore, performing a trend test is considered questionable.

** HCD incidences reported as incidence of animals with histopathological examination of skin lesions performed. It may be assumed highly unlikely that any skin lesions (which might be skin keratoacanthomas) have been missed by the study pathologist, and an assumption of an overall historical control incidence of 1/study (50 animals generally) might be reasonable.

In four out of the six acceptable rat carcinogenicity studies, increased incidences of skin keratoacanthomas were observed in the high dose group. A dose-response relationship was only reported in one of the studies (CA 5.5/010, 1990).

RAC notes that in none of the carcinogenicity studies, were the incidences of skin keratoacanthomas statistically significantly increased in a pairwise comparison based on the statistically analysis included in the study reports (2-sided testing). However, as shown in the table above, when including the p-values for the trend test (Cochran-Armitage) performed by Portier *et al*. (2020, B.6.5.18.2) (1-sided) and by the DS (2-sided) and their respective p-values, statistical significance was reported in the studies CA 5.5/007-009 (1993), CA 5.5/004 (1997), CA 5.5/010 (1990) and CA 5.5/001 (2009) with p-values < 0.05 when using the 1-sided trend test by Portier *et al.* (2020, B.6.5.18.2).

Overall, RAC considers that depending on the statistical method used, the increased incidences of skin keratoacanthomas in male rats were either non-significant, borderline, or significant. However, RAC notes that when performing trend tests, in cases where effects only occur at the highest dose, it is high dose levels that trigger the statistical significance in a trend test. Skin keratoacanthomas were only reported in male rats and not in female rats or male and female mice. The incidences exceeded the available HCD range or from individual studies when available, however, noting that the HCD are very limited for the induction of skin keratoacanthomas in male rats. Skin keratoacanthoma is a benign tumour which is shown to be rather common in aged male rats (Zwicker *et al*., 1992). According to this paper, these tumours are in general first observed at an average age of 549 days (range of 303 - 702 days). In the rat studies with glyphosate, this tumour type was also reported after approximately 550 days (based on the available data for CA 5.5/001, 2009; CA 5.5/003, 1997; CA 5.5/007-009, 1993). Furthermore, it is noted that no malignant squamous cell carcinomas were reported. No plausible underlying mechanism is currently identified for the induction of this tumour type. In humans, this type of benign skin tumours is associated with multiple exposure to sunlight, whereas in rats, which are most likely only exposed to artificial light, the cause of skin keratoacanthomas is unknown. The relationship to exposure to glyphosate is therefore considered unknown. Based on the weight of the evidence, RAC considers that the increase in skin keratoacanthomas only reported in male rats is not of sufficient relevance for classification for carcinogenicity.

Summary of rat long-term/carcinogenicity studies

Seven combined chronic toxicity/carcinogenesis studies in rats are included in the RAC evaluation. Six of these studies are regarded as valid since they are guideline-compliant studies using sufficiently high doses and sufficient numbers of animals per dose group. The study CA 5.5/011 (1981), a low dose study with important reporting deficiencies, is included in the opinion as a supporting study for the evaluation of potential increases in pancreatic adenomas and interstitial cell tumours in the testes. No treatment related reductions in survival were observed in the rat studies. Based mainly on information provided in the CLH dossier and the RAR, and the assessment of the studies by Portier *et al*. (2020, B.6.5.18.2) and Crump *et al.* (2020, B.6.5.18.1),

RAC has evaluated data related to tumours in the pancreas, liver, thyroid, testes, pituitary and the skin reported as skin basal cell tumours and skin keratoacanthomas. RAC notes that the analysis made by Crump *et al.* (2020, B.6.5.18.1) shows that statistically significant effects on tumour incidences should be carefully evaluated for biological relevance due to the high number of studies assessed, as chance findings may occur. Further, RAC notes that in general survival was not reduced even in the high glyphosate dose groups. In some of the rat carcinogenicity studies, the number of animals that survived until the end of the studies was higher in male rats in the top dose versus the control group which could have an influence on the incidence of age-related tumours in these studies (CA 5.5/001, 2009; CA 5.5/002, 2001; CA 5.5/005, 1996).

In male rats, increased incidences of benign pancreatic and liver tumours were reported in the study CA 5.5/010 (1990) with some support for pancreatic islet cell adenoma from the study CA 5.5/011 (1981). Further, in the study CA 5.5/011 (1981) an increased incidence of testes interstitial cell tumours was reported. The increase in pancreatic islet cell adenoma was statistically significant in pairwise testing of the low dose group compared with the control group, but not in the trend test. The increases in liver adenomas were not significant in the pairwise testing but were positive in the trend test (p=0.0171). The increase in testes interstitial cell tumours was reported, however, without a clear dose-response relationship, but was statistically significant in the pairwise testing. However, it was noted that this is a common tumour in aging rats. In addition, CA 5.5/011 (1981) was considered to be a low quality study due to insufficient reporting and the low doses used in the study.

The increase in skin keratoacanthomas was not statistically significantly increased in a pairwise comparison (2-sided testing). However, the incidences were statistically significantly increased when a 1-sided trend test was used but not a 2-sided trend test in the following studies: CA 5.5/007 (1993); CA 5.5/004 (1997); CA 5.5/010 (1990) and CA 5.5/001 (2009).

The CA 5.5/010 (1990) study reported an increase in thyroid C-cell adenoma in males and females. The increased incidences were not significant in males and were only statistically significant in the trend test in females (p=0.0435) and not in pairwise testing versus control.

The CA 5.5/001 (2009) study reported an increase in pituitary adenomas in males and females. The increased incidence showed a statistically significant trend in male and female rats (1-sided, p=0.045 and p=0.014, respectively). However, it was noted that the trend test including the low and mid dose is not considered appropriate since the histopathological examination was only performed on animals that died pre-terminally and that were moribund sacrificed and did not take into account all the animals per dose group.

In a weight of evidence assessment, the significant increases in tumour incidences were observed for benign neoplastic lesions (adenomas) and no evidence that glyphosate induces a progression into more malignant forms were observed for the tumour types evaluated. Furthermore, increased incidences of the pancreatic islet adenomas, the testes interstitial cell tumours, the skin basal cell tumours, the skin keratoacanthomas and the hepatocellular adenomas were only observed in male rats. The incidences of pancreatic islet adenomas were above the historical control range from the test facility, whereas the liver adenoma incidences were within the historical control range and those for the thyroid C-cell adenoma were at the upper range of the HCD.

Limited information was provided to RAC on potential findings in the planned interim sacrificed animals.

RAC has reviewed the rat carcinogenicity studies and has not changed its opinion from 2017. The Committee considers that the rat studies do not provide convincing evidence of glyphosate

induced neoplasia across the seven studies evaluated and therefore does not support classification for carcinogenicity.

Mouse carcinogenicity studies

Study selection - mouse bioassays

The DS included seven carcinogenicity studies in mice; however, two of the studies were considered by the DS and also by RAC to be of unacceptable quality (table 53 of the CLH dossier). Although no indication of carcinogenic potential was observed in either of these unacceptable studies, in both cases the doses were too low and multiple severe deviations from the relevant OECD TG were noted. Therefore, five long-term studies in mice were assessed by RAC for the induction of tumours following exposure to glyphosate, all performed according to OECD TG 451 with four studies in CD-1 mice and one study in Swiss albino mice. These five mice carcinogenicity studies were also assessed in the RAC opinion from 2017. In none of the studies with CD-1 mice, was glyphosate treatment associated with reduced survival. There was a slightly higher mortality in the Swiss albino mice of the high dose group in both males and females.

Three mouse carcinogenicity studies were included in the IARC Monograph (CA 5.5/023, 1983; CA 5.57020-021, 1993; a dermal initiation-promotion study by George *et al*. 2010). The latter study (which was considered by IARC to be inadequate for assessing carcinogenicity of glyphosate) used exposure to a glyphosate-based herbicide and is therefore not evaluated in the current RAC opinion on glyphosate itself. The following three mouse studies evaluated by RAC were not evaluated by IARC: CA 5.5/018-019 (1997); CA 5.5/012-015 (2009) and CA 5.5/016-017 (2001).

Renal tumours, haemangiosarcomas in males, haemangiomas in females, and malignant lymphomas were evaluated by RAC. The evaluation of the mouse cancer studies is mainly based on information provided in the CLH dossier and the RAR (and RAC also had full access to the original study reports).

Renal neoplasms

Table: *Incidences of renal adenomas and carcinomas combined in male mice*

Study [strain]	Control (%)	Low dose (%) [dose]	Mid dose (%) [dose]	High dose (%) [dose]	Fisher's exact test (high dose vs control) Cochran-Armitage trend test
CA 5.5/023[a], 1983 [CD-1]	1 / 49 (2%)	0 / 49 (0%) [157]	1# / 50 (2%) [814]	3## / 50 (6%) [4841]	p=0.617 p=0.0339
CA 5.5/020-021, 1993 [CD-1*]	2# / 50 (4%)	2# / 25 (8%) [100]	0 / 21 (0%) [300]	0 / 50 (0%) [1000]	No significant increase
CA 5.5/018-019, 1997 [CD-1]	0 / 50 (0%)	0 / 50 (0%) [165]	0 / 50 (0%) [838]	2 / 50 (4%) [4348]	p=0.495 p=0.0078
CA 5.5/012-015, 2009 [CD-1]	0 / 51 (0%)	0 / 51 (0%) [71]	0 / 51 (0%) [234]	0 / 51 (0%) [810]	No significant increase
CA 5.5/016-017, 2001 [Swiss albino]	0 / 50 (0%)	0 / 50 (0%) [15]	1 / 50 (2%) [151]	2 / 50 (4%) [1460]	p=0.495 p=0.039

[a] Pathology Working group (EPA, 1986) re-evaluation of kidney lesions, # including one carcinoma, ## including two carcinomas.

* At 100 and 300 mg/kg bw/d only animals that died during the study or that were killed in extremis were investigated. The statistics are based on incidences in 50 animal/dose group instead of the incidence/number of animals investigated.

As noted by the Pathology Working Group (EPA, 1986) in their re-evaluation of the data in the CA 5.5/023 (1983) study, differentiation between tubular cell adenoma and tubular cell carcinoma is not always clearly apparent and both lesions are derived from the same cell type. Accordingly, it is the combined incidences that have been used in the statistical analysis.

Low, but elevated incidences of renal tumours were reported at the high dose exposures in three of the five mouse carcinogenicity studies (table above). The increases in renal tumours were not statistically significant in pairwise comparisons (Fisher's exact test), but when the Cochran-Armitage trend test was used, statistical significance was reported in these studies.

All kidney tumours were observed at termination.

No increase was reported in related preneoplastic lesions (renal tubular hyperplasia or necrosis) in male mice. In study CA 5.5/023 (1983), non-neoplastic kidney pathology in the form of chronic interstitial nephritis was reported to be increased but is not considered to be a precursor for renal tubular cell adenoma. No evidence of a significant reduction in kidney function were reported in these studies.

Renal adenomas and carcinomas are rare tumours in CD-1 mice and Swiss mice. The spontaneous control incidences for CD-1 male mice included for the CA 5.5/018-019 (1997) study were based on seven studies performed between 1993 and 1998 with a mean of 0.28% and a range of 0 - 2%. No HCD was available anymore from the CA 5.5/023 (1983) study. The incidences in the high dose CD-1 mice are slightly outside the control range for renal adenomas/carcinomas in the CA 5.5/018-019 (1997) study. Spontaneous control incidences for Swiss male mice included for the CA 5.5/016 (2001) study were based on eight studies performed between 1996 and 2002, with a mean of 2.0% and a range of 0 - 6%. The increased incidence of renal tubular adenomas in the CA 5.5/016 (2001) study was within the HCD and is therefore considered incidental and not related to glyphosate exposure.

In two of the five studies, no renal tumours were reported at the two highest doses and in two studies, adenomas/carcinomas were reported in the control groups. Furthermore, no increase in renal tumours was reported in female mice. There was a positive trend in male mice, but the findings were not consistent across all studies. RAC notes that although the p-value determined in the trend test in the study CA 5.5/018-019 (1997) indicated that the finding was statistically significant, there were only two adenomas among the 200 males examined in this study.

In two of the three positive studies (CA 5.5/018-019, 1997; CA 5.5/023, 1983), increased tumour incidences were only observed at very high doses (> 4000 mg/kg bw/d) at which the body weight gain in males were decreased compared to controls by up to 11% and 15% in the CA 5.5/023 (1983) and the CA 5.5/018-019 (1997) study, respectively. The OECD TG 452 for carcinogenicity studies does not give a precise top dose recommendation, but states that the highest dose level should normally be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death, and the highest dose level should be chosen to elicit evidence of toxicity, as evidenced by, for example, depression of body weight gain (approximately 10%). RAC therefore gives less weight to the findings at these very high dose levels. RAC also notes that the mouse is exposed to glyphosate via the diet with a high exposure to the gastrointestinal tract. The human relevance of the renal tumours at very high doses is considered to be low and the overall evidence for the increase in renal tumours having been caused by glyphosate is considered insufficient for classification as was also concluded in the RAC opinion from 2017.

An increased incidence of haemangiosarcoma was reported in two studies in male CD-1 mice (see the table below).

Table: *Incidence of haemangiosarcoma in male CD-1 mice*

Dose (mg/kg bw/d)	Haemangiosarcoma (%)	Fisher's exact test	Dose (mg/kg bw/d)	Haemangiosarcoma (%)	Fisher's exact test
CA 5.5/020-021, 1993 (24 months)*			CA 5.5/018-019, 1997 (18 months)		
0	0 / 50 (0%)		0	0 / 50 (0%)	
100	0 / 25 (0%)		165	0 / 50 (0%)	
300	0 / 21 (0%)		838	0 / 50 (0%)	
1000	4 / 50 (8%)	p=0.059	4348	2 / 50 (4%)	p=0.495
Cochran-Armitage trend test	p=0.0004			p=0.0078	

* At 100 and 300 mg/kg bw/d only animals that died during the study or that were killed in extremis were investigated. The statistics are based on incidences in 50 animal/dose group instead of the incidence/number of animals investigated.

Haemangiosarcomas are vascular tumours and were mostly found in liver and spleen. Increased incidences of haemangiosarcomas were reported in high dose animals in the studies CA 5.5/020-021 (1993) and CA 5.5/018-019 (1997). The incidence in the high dose male mice in the CA 5.5/020-021 (1993) study was at the upper edge (8%) of the HCD of the performing laboratory in six studies from 1988 to 1991 (mean incidence 3.3%, range 0 - 8%). No HCD for haemangiosarcoma from the CA 5.5/018-019 (1997) test facility was available to RAC. The 4% incidence at the high dose (greater than 4000 mg/kg bw/d) in the CA 5.5/018-019 (1997) study is within the historical control range for CD-1 mice from Charles River between 1987 to 2000, (Giknis and Clifford, 2005[1]) and showed hemangiosarcoma (whole body) in eight out of 52 studies with a range of 1.67 - 12%.

When pairwise comparison with the Fisher's exact test was used, the increase in haemangiosarcomas reported in the study CA 5.5/018-019 (1997) was not statistically significant. However, when the Cochran-Armitage trend test was used, statistical significance was reported in both studies. RAC notes that although the p-value determined by the trend test in the study CA 5.5/018-019 (1997) indicated that the finding was statistically significant, there were only two tumours among the 200 males examined.

In three of the five studies, no increases in the incidences of haemangiosarcomas were reported in response to glyphosate treatment. Female mice had variable, but low incidences in haemangiosarcomas, with no dose-response relationships. Across both sexes and all five studies, the findings of an increase in haemangiosarcomas in response to glyphosate exposure were inconsistent and the incidences are considered to be within the historical control range and not related to glyphosate exposure. This is the same conclusion as in the RAC opinion from 2017.

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[1] available online at: https://www.criver.com/sites/default/files/resources/SpontaneousNeoplasticLesionsintheCrlCD-1ICRMouseinControlGroupsfrom18Monthto2YearStudies%E2%80%94March2005.pdf

Haemangioma in female mice

Haemangioma in female mice was not assessed in the RAC opinion from 2017. A statistically significant trend for an increased incidence of mesenteric lymph node haemangioma in female Swiss mice (1-sided trend test, p=0.004) and female CD-1 mice (1-sided trend test, p=0.002) was highlighted by Portier *et al*. (2020, B.6.5.18.2) from the CA 5.5/016 (2001) and CA 5.5/018-019 (1997) studies. In the Swiss mice, the incidences were 1/50, 0/50, 0/50, 4/50 in the control, 15, 151 and 1467 mg/kg bw/d groups, respectively. RAC notes that two of the four haemangiomas in the high dose group were reported in the same animal (ovary and mesenteric lymph node). In the CD-1 mice the incidences were 0/50, 0/50, 2/50 and 5/50 in the control, 153, 787 and 4116 mg/kg bw/d groups, respectively. No HCD was provided in the CLH dossier. RAC notes that a statistically significant increase was reported at a very high dose level, and that this tumour type was not reported in male mice in the other carcinogenicity studies nor in the rat studies. RAC therefore considers this finding, in a weight of evidence assessment, as incidental and not related to glyphosate exposure.

Malignant lymphoma

In mice, lymphoma is a common, spontaneously occurring neoplasm. An increased incidence of malignant lymphoma was reported in three carcinogenicity studies in CD-1 mice and one study in Swiss albino mice (see the table below).

Table: *Incidences of malignant lymphoma in male and female mice*

Study; Strain; Duration			Males				Females		
CA 5.5/012-015, 2009 Crl:CD-1; 18 months	Dose (mg/kg bw/d)	0	71	234	810	0	98	299	1081
	Affected	0/51 (0%)	1/51 (2%)	2/51 (4%)	5/51 (10%)	11/51 (22%)	8/51 (16%)	10/51 (20%)	11/51 (22%)
	Fisher's exact test -1-sided* -2-sided**				0.01<p<0.05 p=0.056	No significant increase			
	Cochran-Armitage trend test -1-sided* -2-sided**	p=0.0007 p=0.0037							
CA 5.5/018-019, 1997 Crj:CD-1; 18 months	Dose (mg/kg bw/d)	0	165	838	4348	0	153	787	4116
	Affected	2/50 (4%)	2/50 (4%)	0/50 (0%)	6/50 (12%)	6/50 (12%)	4/50 (8%)	8/50 (16%)	7/50 (14%)
	Fisher's exact test-- 1-sided* -2-sided**				p=0.269	No significant increase			
	Cochran-Armitage trend test -1-sided* -2-sided **	p=0.016 p=0.0085							

Study; Strain; Duration		Males				Females			
CA 5.5/020-21, 1993 CD-1 (sub-strain not specified); 24 months	Dose (mg/kg bw/d)	0	100[##]	300[##]	1000	0	100[##]	300[##]	1000
	Affected[#]	4/50 (8%)	2/25 (8%)	1/21 (4%)	6/50 (12%)	14/50 (28%)	12/34 (35%)	9/24 (38%)	13/50 (36%)
	Fisher'exact test -1-sided* -2-sided**				p=0.741	No significant increase			
	Cochran-Armitage trend test -1-sided* -2-sided**	p=0.087 p=0.076							
CA 5.5/023[a] 1983 Crl:CD-1; 24 months	Dose (mg/kg bw/d)	0	157	814	4841	0	190	955	5874
	Affected	2/48 (4%)	5/49 (10%)	4/50 (8%)	2/49 (4%)	6/50 (12%)	6/48 (13%)	7/49 (14%)	11/49 (22%)
	Fisher' exact test -1-sided* -2-sided**	No significant increase				No significant increase			
	Cochran-Armitage test -1-sided* -2-sided**								
CA 5.5/016, 2001; Swiss albino	Dose (mg/kg bw/d)	0	15	151	1460	0	15	151	1460
	Affected	10/50 (20%)	15/50 (30%)	16/50 (32%)	19/50 (38%)	18/50 (36%)	20/50 (40%)	19/50 (38%)	25/50 (50%)
	Fisher's exact test -1-sided* -2-sided**				0.01<p<0.05 p=0.077				p=0.225
	Cochran-Armitage trend test -1-sided* -2-sided**	p=0.064 p=0.065				p=0.07 p=0.068			
	Peto-test*** -1-sided -2-sided	p=0.046 p=0.092				p=0.087 p=0.174			

* From Portier *et al.* (2020, B.6.5.18.2); ** From RAC opinion 2017; *** From DS

Based on histological examination of lymph nodes with macroscopic changes.

At 100 and 300 mg/kg bw/d only animals that died during the study or that were killed in extremis were investigated. The statistics are based on incidences in 50 animal/dose group instead of the incidence/number of animals investigated.

[a] Lymphoreticular neoplasms (total); malignant lymphoma not used as a separate entity. Three cases of granulocytic leukaemia (not lymphoma) in low dose group.

RAC also noted the study in mice (Anonymous, 1999) reported in the 2016 evaluation by JMPR. Only female mice were included, and the study reported an increased incidence of malignant lymphoma (6%, 8% and 12% in the control, mid and high dose group, respectively). However, it was noted that the high dose was 8690 mg/kg bw/d and no HCD were provided. The results were not statistically significant with a Fisher exact test (pairwise comparison, JMPR, 2016) as well as in a trend test (1-sided, Portier *et al.,* 2020, B.6.5.18.2, p=0.05 and 2-sided p > 0.05, JMPR, 2016).

When pairwise comparison with Fisher's exact test was used, the increases in lymphomas did not reach statistical significance in any of the studies using the 2-sided test. However, Portier *et al.*

(2020, B.6.5.18.2) also assessed the results by using a 1-sided test and found a statistically significant increase in male mice in the CA 5.5/016 (2001) and CA 5.5/012-015 (2009) studies. In two of the studies in CD-1 mice (CA 5.5/018-019, 1997; CA 5.5/012-015, 2009), a statistically significant trend (2-sided) for malignant lymphoma was observed in male animals when using the Cochran-Armitage trend test.

No significant increases in malignant lymphomas were found in the CA 5.5/023 (1983). In this study, malignant lymphoma was not used as a separate histopathological entity and RAC notes that three cases of granulocytic leukaemia were reported in the low dose group. However, the term "lymphoreticular neoplasms" is considered to include the group of malignant lymphomas and the findings were reported to be non-significant in the RAR.

The tumour incidence of 12% at the high dose of 4348 mg/kg bw/d in the study by CA 5.5/018-019 (1997) was within the historical control range for Crj:CD-1 male mice obtained from seven studies. The range was 3.8% to 19.2% with a mean of 7%. However, it was noted that six of the seven studies had a control incidence ≤ 6% leading to a range of 3.8% to 6% with a mean of 4.92%. Therefore, when taking into account HCD from the six studies the incidences of malignant lymphoma in male mice exceeded the HCD.

The 10% incidence in the study CA 5.5/012-015 (2009) was borderline significant in the pairwise Fisher's exact test. However, the incidence of lymphomas in controls was very low and there were limited HCD available from the laboratory. In a trend test (1- and 2-sided), a statistically significant increase was reported for both.

There was no significant increase in malignant lymphomas in the study CA 5.5/020-021 (1993). It should be noted that only those lymph nodes which showed macroscopic changes were investigated histologically. This may lead to an underestimation of the actual tumour numbers. No HCD from the test facility were identified. It should also be noted that the sub-strain of CD-1 mice used in the study CA 5.5/020-021 (1993) is not known and the data should be used with caution.

In Swiss albino mice (CA 5.5/016, 2001), the incidence of malignant lymphoma in male and female mice at the top dose was 38% and 50%, respectively. However, the high background incidence in this strain must be taken into consideration. The HCD in males had a mean of 15.8% with a range of 6 - 30% and in females a mean of 33% with a range of 14 - 58%. Thus, the incidences of malignant lymphomas were above the upper range of the HCD for the male mice.

No significant increases in malignant lymphomas were found in the mouse studies when assessed by the pairwise Fisher's exact test (2-sided), but when a 1-sided test was used a significant increased incidence was found in study CA 5.5/012-015 (2009) and CA 5.5/016 (2001) by Portier et al. (2020, B.6.5.18.2). However, in two of the five studies (CA 5.5/012-01,5 2009; CA 5.5/018-019, 1997), a significant positive trend for malignant lymphoma incidences in males was reported. In one study (CA 5.5/016 (2001)) using a Peto-analysis (1-sided) a significantly increased incidence was seen. In two studies (CA 5.5/020-021 (1993) and CA 5.5/023 (1983)), increases were observed that were not statistically significant. In the oldest of the studies (CA 5.5/023 (1983)), the term malignant lymphoma was not used, but there was no statistically significant increase in lymphoreticular neoplasms reported in this study in response to glyphosate exposure. Thus, the lymphoma incidences in male mice show a slight, but clearly variable increase. The biological and human relevance of the findings is uncertain for the following reasons:

  i.   the maximum incidences in the majority of the studies were considered to be within the historical control range for the CD-1 mice, although adequate HCD were not available for all studies;

ii. the increases in malignant lymphoma incidences appeared to be confined to the high dose groups in the CD-1 mice;

iii. the incidence of malignant lymphomas is known to be related to the age of the animals. However, significant associations between exposure to glyphosate and induction of malignant lymphomas were not observed in the 24-month studies. Furthermore, there was no reduction in overall survival in the exposed groups;

iv. no parallel increases were observed in female CD-1 mice. It is known that female CD-1 mice are usually more prone to develop spontaneous malignant lymphoma than male mice (Son and Gopinath, 2004). The lymphoma incidences were generally higher in females than in males, but no glyphosate related increases were seen in female CD-1 mice.

RAC has reviewed all of the data and in a weight of evidence assessment concludes that the reported incidences of malignant lymphoma in CD-mice and Swiss mice are not considered related to glyphosate exposure, which is in agreement with the RAC opinion from 2017.

Summary of mouse carcinogenicity studies

Five mouse carcinogenicity studies are included in the RAC evaluation. All these studies are regarded as valid since they are considered to be guideline compliant (four are also GLP compliant) and all used sufficiently high doses and sufficient number of animals. No treatment-related reductions in survival were observed in these studies. Based mainly on information provided in the CLH dossier and the RAR, RAC has evaluated data related to kidney tumours, haemangiosarcomas, haemangioma and malignant lymphomas. RAC notes that the analysis made by Crump *et al.* (2020, B.6.5.18.1) shows that statistically significant effects on tumour incidences should be carefully evaluated for biological relevance due to the high number of studies assessed as chance findings may occur.

An increase in renal neoplasms (adenomas and carcinomas combined) was reported in males at the top doses in three of the five studies. Furthermore, an increase in haemangiosarcoma was reported in CD-1 males at the top doses in two of the studies, and an increased incidence of malignant lymphoma was reported in three carcinogenicity studies in CD-1 male mice and one study in Swiss albino male mice. In females, the only significant finding was an increased incidence of haemangioma in the study using Swiss mice (CA 5.5/016, 2001) and in one study with CD-1 mice (CA5.5/018-19, 1997). This is a benign tumour type, and it was not reported in female mice in the other carcinogenicity studies.

The observed increases in tumour incidences were all non-significant in pairwise comparisons with control groups by the Fisher's exact test (2-sided). However, several of the findings were positive when tested using the Cochran-Armitage trend test. In two of the studies (CA 5.5/016, 2001; CA 5.5/018-019, 1997), tumours were observed at multiple sites in males in the top dose groups.

All tumours were observed at termination and RAC has no information concerning any possible reduction in tumour latency. However, for the renal adenomas there was no evidence for a progression to malignancy in two of the studies, whereas the data for the third study (CA 5.5/023, 1983) were equivocal.

The high dose levels in two of the five mouse studies (CA 5.5/018-019, 1997; CA 5.5/023, 1983) exceeded 4000 mg/kg bw/d and the body weight gain in males in the high dose group was decreased by more than 15% compared to controls in the CA 5.5/018-019 (1997) study,

suggesting that the doses used were excessive (OECD TG 451 and 116)[1]. RAC notes that the biological relevance of the slight increases in tumours in these two studies are considered equivocal since they were seen only at the top doses.

In mice, the incidences of renal neoplasm and haemangiosarcomas were increased only in males and haemangiomas in females. Malignant lymphoma was present in both male and female mice reflecting that this is a very common spontaneous neoplasm in mice. However, only in the Swiss albino mice a glyphosate associated increase in this tumour type in females was observed. There are no toxicokinetic data to RAC's knowledge in support of significant differences in ADME between male and female mice; thus, the mostly negative findings in female CD-1 mice were regarded as a sign of low consistency of the mouse carcinogenicity data.

All the five studies reported a positive trend in males for one or more of the tumour types evaluated, suggesting a potential concern for a tumour effect at high glyphosate doses. However, in a weight of evidence assessment, in the cases where increased tumour incidences were found in the high dose groups, the incidences were either within or slightly above the range of HCD or spontaneous incidence levels reported for CD-1 mice. Furthermore, the apparent sex differences in response remain unexplained and this lowers the consistency of the reported findings in mice as well as increasing the inconsistency in tumour incidences between the mouse carcinogenicity studies. The increased tumour incidences observed are therefore considered to be of equivocal biological relevance.

RAC has thoroughly reviewed the mouse carcinogenicity studies based on the proposal by DS and retains its opinion on classification from 2017. RAC considers that the mouse studies did not demonstrate convincing evidence of glyphosate induced neoplasia across the five studies evaluated, and therefore does not support classification for carcinogenicity.

A number of organisations, international (WHO/JMPR), EU (EFSA) and national (for example US EPA, FSC Japan, PMRA Canada) have assessed the carcinogenic potential of glyphosate. So far, only IARC has concluded that glyphosate is carcinogenic (and genotoxic). Therefore, a detailed comparison of the carcinogenicity evaluation conducted by IARC and RAC is provided below.

Comparison with the IARC evaluation

There is a high degree of similarity between the IARC and the CLP criteria for carcinogenicity classification. However, under the CLP Regulation, where the criteria cannot be applied directly to available identified information, there is an obligation to "*… carry out an evaluation by applying a weight of evidence determination using expert judgement …",* which involves "*… weighing all available information having a bearing on the determination of the hazards of the substance …".*

IARC (monograph 112) states in their rationale for classifying glyphosate in Group 2A: "*In addition to limited evidence for the carcinogenicity of glyphosate in humans sufficient evidence for the carcinogenicity of glyphosate in experimental animals, there is sufficient evidence in animals for carcinogenicity of glyphosate*".

The definition of sufficient evidence of carcinogenicity (common to both IARC and CLP) is that: "*a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a)*

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[1] According to OECD TG 451 the maximum dose should result in a "depression of body weight gain (approximately 10%)". Furthermore, according to OECD GD 116, the MTD is … "conventionally defined as the highest dose to produce toxic effects without causing death and to decrease body weight gain by no more than 10% relative to controls".

two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well-conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence. A single study in one species and sex might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites;"

The IARC Monograph states, concerning the studies in rats: *"For the five feeding studies in rats, two studies in the Sprague-Dawley strain showed a significant increase in the incidence of pancreatic islet cell adenoma in males – one of these two studies also showed a significant positive trend in the incidences of hepatocellular adenoma in males and of thyroid C-cell adenoma in females. Two studies (one in Sprague-Dawley rats, one in Wistar rats) found no significant increase in tumour incidence at any site."*

The IARC Monograph states, concerning the studies in mice: *"There was a positive trend in the incidence of renal tubule carcinoma and of renal tubule adenoma or carcinoma (combined) in males in one feeding study in CD-1 mice. Renal tubule carcinoma is a rare tumour in this strain of mice. No significant increase in tumour incidence was seen in female mice in this study. In the second feeding study, there was a significant positive trend in the incidence of haemangiosarcoma in male CD-1 mice. No significant increase in tumour incidence was seen in female mice in this study."*

It is noted that the evaluation performed by RAC is based on a larger experimental database than the IARC evaluation as presented in the CLH dossier (nine vs five rat studies and five vs two mouse studies, respectively).

In contrast to IARC, RAC does not consider that a genotoxic mode of action has been demonstrated for glyphosate (see preceding section on germ cell mutagenicity).

Mechanistic studies from public literature

RAC notes the studies included by the DS assessing the possible mode of action following exposure to glyphosate. These included one *in vivo* study in WT mice and Vk*MYC mice assessing the effect of exposure to glyphosate and multiple myeloma, one *in vitro/in vivo* study addressing renal tubular toxicity and the induction of oxidative stress, and four *in vitro* studies assessing epigenetic effects, autophagic effects and lipid accumulation following exposure to glyphosate. The studies are presented in table 55 in the CLH dossier.

In the *in vivo* study by Wang *et al*. (2019) WT mice and Vk*MYC mouse (multiple myeloma, animal model) were exposed to glyphosate, 1 g/L in drinking water for 72 weeks (corresponding to 90 mg/kg bw/d). The study reported more severe effects from glyphosate in the Vk*MYC mouse compared to the WT mouse on survival, spleen weight, IgG levels and haematology. In WT mouse, glyphosate induced benign monoclonal gammopathy (mouse equivalent to monoclonal gammopathy of undetermined significance in humans) and promoted multiple myeloma progression in Vk*MYC mouse. RAC notes that the study has some limitations, including the low number of animals for some endpoints and only one dose tested. However, the effects reported on haematological parameters in both WT and transgenic mice in the study by Wang and co-workers (Wang *et al*., 2019) do not appear to be a consistent treatment related finding in the mouse cancer bioassays. RAC agrees with the DS that the study does not have any direct impact on the overall assessment for glyphosate.

The study by Gao *et al*. (2019) examining potential mechanisms of kidney toxicity is also described under STOT RE and germ cell mutagenicity. The study examined effects of glyphosate on a human renal proximal tubular epithelial cell line (HK-2) as well as in male ICR mice exposed

for 28 days to 400 mg/kg bw/d of glyphosate. In the HK2 cells, an increase in ROS and oxidative stress (MDA levels) was reported at concentrations ≥ 40 µM, concentrations that also induced increased cell death. In the *in vivo* experiment (6 animals/group), exfoliation of renal tubular cells as well as increased oxidative stress and increase in the percentage of apoptotic cells in the kidney was observed in the absence of change in relative kidney weights. Further, an increase in urinary levels of β2-microglobulin was reported, indicating a reduction in kidney function. Activation of NMDA receptor signalling by glyphosate was proposed by the authors as a potential mechanism for induction of oxidative stress in the kidney. The relationship of ROS and oxidative stress induction in selected tissues and genotoxicity is discussed in more detail in the germ cell mutagenicity section.

Two recent studies from the same group examined potential epigenetic changes following glyphosate exposure *in vitro* (Kwiatkowska *et al*., 2017, CA 5.4/008; Wozniak *et al*., 2020). In human peripheral blood mononuclear cells, glyphosate reduced global DNA methylation levels and altered DNA methylation in promotor regions of P21 and P53, with no changes in P16, BCL2 and CCND1. The gene expression was decreased for P16 and P53 and increased for BC12, CCND1 and P12. RAC notes that no clear dose-responses were reported for these changes and the changes in DNA methylation profile were minimally correlated with gene expression (Wozniak *et al*., 2020).

Duforstel *et al*. (2019) assessed the DNA methylation pattern in non-neoplastic MCF10A cells and reported that low doses of glyphosate (10^{-11} M) induced DNA hypomethylation with TET3 overexpression. The study concluded that glyphosate may promote development of mammary tumours but is not considered as oncogenic. RAC notes that since only one low concentration of glyphosate was tested, no dose-response relationship could be determined.

Whereas necrosis may promote cancer development, apoptosis and autophagy are considered protective. Increased oxidative stress is a recognised mechanism by which non-DNA reactive chemicals may induce oxidative DNA-lesions. Inadequate repair of such lesions may in turn cause increased mutations and CA if not repaired (Smith *et al*., 2016). ROS and oxidative damage to macromolecules, including DNA, also occurs in normal physiology and in several pathological conditions not associated with increased cancer risk. As discussed in the germ cell mutagenicity section, the evidence that glyphosate induces mutations is very weak. Furthermore, although an increase in the incidences of renal tumours were reported at the high dose exposures in three of the mouse studies, no related increase in preneoplastic lesions or impairment of kidney function were reported in support of a treatment related effect.

Human data – epidemiological studies

In the epidemiological studies described below, the data relate to exposure to glyphosate-based herbicide, not specifically to glyphosate. The exact expressions used in the original studies vary. In this section, the term "glyphosate-based herbicide" is used, regardless of what terminology was used in each of the individual epidemiological articles described. Overview tables (see tables 54 of the CLH report including reliable studies, studies reliable with restrictions and studies of low reliability as well as table 2.6.5 of the CLH report which includes studies where the DS has identified a data gap). Many of the studies are interlinked and are used in the reviews, meta-analyses etc. Some additional publications were brought forward in the consultation and are included below as well. RAC notes that exposure to Roundup® – a glyphosate-based herbicide - has occurred in agriculture since 1974 (U.S.), and later to other glyphosate-based herbicides. The use of glyphosate-based herbicides increased massively, especially in the US after the introduction of genetically modified glyphosate-tolerant crops in 1996.

Available epidemiological studies generally consist of cohort studies and case-control studies[1] on cancer, as well as reviews, re-analyses/pooled analyses, systematic reviews, and meta-analyses of the studies mentioned above. No other source of human data is available apart from epidemiological studies. Findings of non-Hodgkin's Lymphoma, multiple myeloma and acute myeloid leukaemia are of particular interest in the CLH dossier and are also the focus of this opinion, but other lymphomas and leukaemias, and other cancer types have also been studied. RAC notes that non-Hodgkin's Lymphoma is not a specific disease, but a broad spectrum of disorders more correctly referred to as lymphocytic lymphomas, each with possible different aetiologies. They are all classified as not being Hodgkin's Lymphoma, and the terminology has changed over the years - some lymphomas are described differently today compared to the past. This complicates the evaluation of the studies.

Cohort study

The US Agricultural Health Study (AHS)

A single large prospective cohort study is available, which enrolled 57311 private and commercial applicators (farmers/registered pesticide applicators, and in addition spouses and children, in total 75000 participants from Iowa and North Carolina) (De Roos *et al*., 2005). The study was initiated by the National Cancer Institute (NCI) in cooperation with the National Institute of Environmental Health Sciences (NIEHS), National Institute for Occupational Safety and Health (NIOSH) and EPA in 1993. The study design was described by Alavanja *et al*. (1996), later reported by De Roos *et al*. (2005), and with a recent update by Andreotti *et al*. (2018) with more than 11 years follow up compared to the study by De Roos *et al*. (2005) and more than four times the number of glyphosate-based herbicide-exposed cancer cases (n=5779 compared with n=1324). The study is still ongoing. The exposure assessment was initially planned to be based on interviews and questionnaires (e.g., on frequency – days of use of pesticides/year - and duration – years of use of pesticides) but also on actual measurements of exposure/environmental and biological monitoring (in 200 families in the cohort). The AHS cohort was evaluated by IARC to be the only cohort study to date to have published findings on exposure to pesticides and the risk of cancer at many different sites and is considered by RAC to have a the most balanced assessment of the association of exposure to glyphosate-based herbicide and the risk of cancer, with a due consideration of bias or confounding factors. Several additional epidemiological analyses, such as nested case-control studies[2], have been carried out and published based on this cohort. It was noted in the study by De Roos *et al*. (2005) that even if the number of participants in the AHS cohort is large, it would have had to be even larger in order to contribute a sufficient number of cases of rare cancers, such as multiple myeloma (32 cases found) to obtain significant results. Further, there were 92 cases of non-Hodgkin's Lymphoma after a follow up time of 6-7 years which did not identify an increased risk, as described below. Age, smoking, other pesticides, alcohol consumption, family history of cancer and education were considered as potential confounders by De Roos *et al*. (2005) and by Andreotti *et al*. (2018). RAC notes that the individual exposure time is longer than the follow up time, as the exposure probably preceded the start of the study (no information reported on actual

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[1] In cohort studies the people are prospectively followed and with a view to determining whether those exposed to a substance develop a disease more frequently that those who have not been exposed. In a case-control study, the exposure in cases in which people have a particular disease are compared retrospectively with those who do not have the disease. In both cases the intention is to establish whether exposure has had a role in development of the disease.

[2] In the nested case-control study, cases of a disease that occur in a defined cohort are identified and, for each, a specified number of matched controls is selected from among those in the cohort who have not developed the disease by the time of disease occurrence in the case.

exposure length or latency time from start of exposure to end of follow up). The cancer cases, such as non-Hodgkin's Lymphoma, were identified as soon as possible after diagnosis and investigated using nested case-control studies[1].

The strengths of this prospective cohort study are that the collection of exposure information was done at the start of follow up (thus independent of health status in order to avoid recall bias), the control of confounders like the use of other pesticides, even investigating the exposure-response relationship and the absence of any proxy respondents.

Case-control studies

Other study populations

There are also other populations besides the one contained in the AHS cohort where the relationship between exposure to glyphosate-based herbicide and the risk of non-Hodgkin's Lymphoma, multiple myeloma and other cancer types have been studied. These are all case-control studies from various regions: Sweden (Hardell and Eriksson, 1999; Hardell *et al*., 2002; Eriksson *et al*., 2008), Australia (Fritschi *et al*., 2005), Canada (McDuffie *et al*., 2001; Pahwa *et al*., 2012; Kachuri *et al*., 2013), Midwestern United States (Iowa and Minnesota, Kansas, Nebraska, by De Roos *et al*., 2003, analysing Cantor, 1992; Hoar, 1986; Zahm, 1990), North America Pooled Project from Iowa, Nebraska and Canada (Presutti *et al*., 2016; Pahwa *et al*., 2019), Italia (Meloni *et al*. (2021) and France (Orsi *et al*., 2009). The Australian study does not report on glyphosate itself ("other herbicides - mainly glyphosate and carbamates") and is not discussed further. A European multi-centre lymphoma case-control study (Cocco *et al*., 2013) was performed in 6 European countries (ES, FR, DE, IE, IT, CZ).

The case-control studies have a retrospective design, which introduces the possibility of recall bias among the participants that can influence the observed risk estimates. Proxy respondents are often used for subjects that have died or become incapacitated, adding further possibilities for bias and misclassification of exposure. RAC notes that as the use of pesticides is typically seasonal and occasional and often involves several pesticides, the retrospective assessment of such exposures, having occurred years or decades earlier, is prone to inaccuracies due to the participants recollection of use of glyphosate-based herbicides, use of other pesticides, exposure duration and use of personal protective equipment.

Statistical associations

Statistical null associations – solid tumours, leukaemia and Hodgkin's lymphoma

No association was found between exposure to glyphosate-based herbicide and the risk of solid tumours, and Hodgkin's Lymphoma (De Roos *et al*., 2005; Engel *et al*., 2005; Flower *et al*. 2004, Koutros *et al*., 2011; Lee *et al*., 2004; Andreotti *et al*., 2009 and 2018; Band *et al*., 2011; Pahwa *et al*., 2011). No association between exposure to glyphosate-based herbicide and increased risk of leukaemia has been found; this was recently supported by Chang and Delzell (2016) in a meta-analysis of De Roos *et al*. (2005). Chang and Delzell (2016) also investigated the risk of Hodgkin's Lymphoma based on the studies by Karunanayake *et al*. (2011) and Orsi *et al*. (2009) and found statistically null associations with Hodgkin's Lymphoma. RAC notes however, that in the recent update of the AHS cohort by Andreotti *et al*. (2018) an increased risk for acute myeloid leukaemia was reported in the highest quartile of exposure and when a 20-year lag period was taken into account.

In relation to other cancer types, Mink *et al*. (2012) reviewed the quality of the following 7 cohort studies (nested case-control studies) all based on the AHS cohort: Flower *et al*. (2004, childhood cancer), De Roos *et al*. (2005, multiple cancer endpoints), Alavanja *et al*. (2003, prostate cancer), Engel *et al*. (2005, breast cancer), Lee *et al*. (2007, colorectal cancer), Andreotti *et al*. (2009,

pancreatic cancer) and Dennis *et al*. (2010, cutaneous melanoma). Mink *et al*. (2012) stated that all of the studies were prone to bias, measurement error, and/or confounding factors, and concluded that with a cautious interpretation of the few positive associations reported in the literature, the epidemiological data considered together do not support a causal association between glyphosate-based herbicide exposure and cancer. No meta-analysis was performed as the authors did not consider it appropriate to calculate quantitative summary relative risk estimates across studies evaluating different site-specific cancers.

In addition, Multigner *et al*. (2008) and Band *et al*. (2011) reported a lack of association between glyphosate-based herbicide use and prostate cancer in case-control studies. Carreon *et al*. (2005) reported a lack of association between gliomas and farm pesticide exposure in women. However, Lee *et al*. (2005) reported an association between glyphosate-based herbicide use and primary adult gliomas, with the odds ratio differing between self-respondents (OR=0.4, 95% CI: 0.1 - 16) and proxy respondents i.e., spouses or first-degree relatives (OR=3.1, 95% CI: 1.2 - 8.2). RAC notes the higher positive associations reported for proxy respondents with glyphosate-based herbicide and several other pesticides, and that this could be related to information bias due to a more accurate reporting of proxies for cases and underreporting by proxies for controls, and therefore no clear conclusion can be drawn from this study.

Landgren *et al*. (2009) reported a lack of association between monoclonal gammopathy of undetermined significance (MGUS, a condition that is sometimes a precursor to multiple myeloma) using data from the AHS cohort. The OR for MGUS for glyphosate-based herbicide users versus non-users, adjusted for age and education level, was 0.5 (95% CI: 0.2 - 1.0).

RAC agrees with the DS that there is no epidemiological evidence of an association between exposure to glyphosate-based herbicide and the risk of solid tumours and Hodgkin's Lymphoma among the studies presented in the CLH dossier.

Statistical associations – Acute Myeloid leukaemia

No association between exposure to glyphosate-based herbicide and acute myeloid leukaemia was reported in the AHS cohort by De Roos *et al*. (2005). However, in the recent update of the AHS cohort by Andreotti *et al*. (2018) an increased rate ratio (RR) for acute myeloid leukaemia was reported in the highest quartile of exposure (RR=2.44, 95% CI: 0.94 - 6.32, p-trend=0.11). The effect was not statistically significant, although the RR was significant for the third quartile of exposure when a 20-year lag period was taken into account (RR=2.04, 95% CI: 1.05 - 3.97, p-trend=0.04) (no assessment of the fourth quartile and 20-year lag-period). It was noted that a low number of cases was included in this subgroup (n=15). When including a 5-year lag period and the highest quartile of exposure the RR was 2.32 (95% CI: 0.98 - 5.51, p-trend=0.07) and included 18 cases. It was noted that about 37% of the participants in the recent update by Andreotti *et al*. (2018) did not complete the follow up questionnaire. For these participants a data-driven multiple imputation procedure was used to impute pesticide use since enrolment (Heltshe *et al*., 2012). Factors used to impute pesticide use included demographic data and medical history, as well as factors related to farm characteristics and reported pesticide use at enrolment. RAC noted that Andreotti *et al*. (2018) performed sensitivity analyses including only participants who completed both the enrolment questionnaire and the follow up questionnaire. For acute myeloid leukaemia there was a non-statistically significant risk when the analysis was limited to participant who completed both questionnaires (RR=2.64, 95% CI: 0.78 - 6.86, p-trend=0.18). RAC further notes that an association between glyphosate-based herbicide exposure and acute myeloid leukaemia has not been previously reported in other epidemiological studies, and that occupational farming and general pesticide exposure have long been linked to the induction of leukaemia (Blair and Freeman, 2007). Further, RAC notes that the latent period between relevant exposure and acute myeloid leukaemia diagnosis is unknown, and it may vary by type of exposure and population characteristics (Linet *et al*., 2006). The association between

acute myeloid leukaemia and exposure to glyphosate should be followed in further updates of the AHS cohort.

Table: *Incidence of acute myeloid leukaemia in relation to intensity-weighted lifetime days of glyphosate-based herbicide use in AHS cohort (from Andreotti et al., 2018)*

Glyphosate use*	Number of acute myeloid leukaemia	RR (95% CI)	P-trend
None	9	1.00 (reference)	
Q1	13	1.62 (0.60 - 4.38)	
Q2	14	1.70 (0.61 - 4.73)	
Q3	12	1.46 (0.49 - 4.37)	
Q4	18	2.44 (0.94 - 6.32)	0.11

* Categorising cumulative lifetime days and intensity-weighted lifetime days of exposure to glyphosate-based herbicide into quartiles: Q1: 1 - 13.74, Q2: 13.75 - 38.74, Q3: 38.75 - 108.4, Q4: ≥ 108.5.

Statistical associations – non-Hodgkin's Lymphoma and multiple myeloma

In the AHS cohort reported by De Roos *et al*. (2005) no association between exposure to glyphosate-based herbicide and the risk of non-Hodgkin's Lymphoma was found. In this study 92 cases of non-Hodgkin's Lymphoma were observed during a median follow up time of 6.7 years, with a RR of 1.1, 95% CI: 0.7 - 1.9 adjusted for age, demographic and life-style factors and exposure to other pesticides. Glyphosate-based herbicide exposure was not associated with non-Hodgkin's Lymphoma incidence overall or with any of the non-Hodgkin's Lymphoma cancer subtypes studied. No dose-response relationship was observed between non-Hodgkin's Lymphoma incidences and cumulative exposure days or intensity-weighted exposure days of glyphosate-based herbicide use. In the recent update of the AHS cohort by Andreotti *et al*. (2018) with an extended follow up of 17.5 years, also no association between exposure to glyphosate-based herbicides and the risk of non-Hodgkin's Lymphoma (575 cases) was found. In the high exposure quartile (> 108 days of glyphosate-based herbicide use) the RR was 0.87, 95% CI: 0.64 - 1.20, p-trend: 0.95 including 440 exposed cases and including the same adjustment factors as in the De Roos *et al*. (2005) study. Andreotti *et al.* (2018) also found no evidence for associations with glyphosate-based herbicide use for any of the non-Hodgkin's Lymphoma subtypes.

Table: *Incidence of non-Hodgkin's Lymphoma in relation to intensity-weighted lifetime days of glyphosate-based herbicide use in AHS cohort (from Andreotti et al., 2018)*

Glyphosate use*	Number non-Hodgkin's Lymphoma	RR (95% CI)	P-trend
None	135	1.00 (reference)	
Q1	113	0.83 (0.59 - 1.18)	
Q2	104	0.83 (0.61 - 1.12)	
Q3	112	0.88 (0.65 - 1.19)	
Q4	111	0.87 (0.64 - 1.20)	0.95

* Categorising cumulative lifetime days and intensity-weighted lifetime days of exposure to glyphosate-based herbicide into quartiles: Q1: 1 - 13.74, Q2: 13.75 - 38.74, Q3: 38.75 - 108.4, Q4: ≥ 108.5.

In the AHS cohort reported by De Roos *et al*. (2005) there was, however, a suggested association with multiple myeloma incidence that the authors recommended to be followed up as more cases occur in the AHS, with a reported RR of 2.6 (95% CI: 0.7 - 9.4) (the most fully adjusted, De Roos *et al*. 2005). However, in the recent update of the AHS cohort by Andreotti *et al*. (2018) with an extended follow up of 17.5 years, no association with multiple myeloma incidences and exposure to glyphosate-based herbicides was reported. The RR was 0.87, 95% CI: 0.45 - 1.69, p-trend 0.84 in the high exposure quartile and included 88 exposed cases. RAC notes that the

lifetime use of glyphosate-based herbicides was ascertained based on two questionnaires, one at enrolment and another follow up questionnaire about 5 years after enrolment. However, about 37% of the participants in the recent update by Andreotti *et al*. (2018) did not complete the follow up questionnaire. For these participants a data-driven multiple imputation procedure was used to impute pesticide use since enrolment as described for acute myeloid leukaemia. RAC further notes that Andreotti *et al*. (2018) also performed sensitivity analyses including only participants who completed both the enrolment questionnaire and the follow up questionnaire, and those results were similar to the results of the full cohort (RR=0.90, 95% CI: 0.63 - 1.27, p-trend=0.54).

Table: *Incidence of multiple myeloma in relation to intensity-weighted lifetime days of glyphosate-based herbicide use in AHS cohort (Andreotti et al., 2018)*

Glyphosate use*	Number multiple myeloma	RR (95% CI)	P-trend
None	30	1.00 (reference)	
Q1	19	0.70 (0.36to 1.36)	
Q2	26	0.94 (0.50 to 1.76)	
Q3	19	0.78 (0.39 to 1.56)	
Q4	24	0.87 (0.45 to 1.69)	0.84

* Categorising cumulative lifetime days and intensity-weighted lifetime days of exposure to glyphosate-based herbicide into quartiles: Q1: 1 - 13.74, Q2: 13.75 - 38.74, Q3: 38.75 - 108.4, Q4: ≥ 108.5.

Leon *et al*. (2019) studied the relationship of 'ever use' of glyphosate-based herbicide with non-Hodgkin's Lymphoma overall or major subtypes in a pooled analysis of three agricultural worker cohorts, from France (n=181747, AGRICAN), Norway (n=147134, CNAP) and US (n=57311, AHS cohort) and estimated cohort-specific hazard ratios (HRs). The exposure was assessed in the AGRICAN and CNAP cohorts by using country-specific crop-exposure matrices (CEMs) to estimate ever exposure to glyphosate. In the AHS cohort self-reported ever application of glyphosate was used to assess ever exposure. The authors reported the association between all of the pre-selected 14 chemical groups and 33 active ingredients with non-Hodgkin's Lymphoma overall and the four most frequent subtypes and acknowledged this resulted in a large number of comparisons. Focusing on glyphosate-based herbicide, the authors concluded that there was no association for non-Hodgkin's Lymphoma overall or for most subtypes, however, they found an association with diffuse large B-cell lymphoma and exposure to glyphosate-based herbicides (ever vs never) with a meta-HR=1.36 (95% CI: 1.00 - 1.85). The cohort-specific HRs for ever use of glyphosate-based herbicide and diffuse large B-cell lymphoma were for AGRICAN HR=1.06 (95% CI: 0.51 - 2.19), CNAP HR=1.67 (95% CI: 1.05 - 2.65) and AHS HR=1.20 (95% CI: 0.72 - 1.98), based on 28, 100 and 93 glyphosate-based herbicide exposed cases, respectively. RAC notes that the meta-HR for glyphosate-based herbicide was higher than the RR from the AHS publication by Andreotti *et al*. (2018) (Q1: RR=1.11, 95% CI: 0.60 - 2.07; Q2: RR=0.94, 95% CI: 0.49 - 1.80; Q3: RR=1.13, 95% CI: 0.59 - 2.17; Q4: RR=0.97, 95% CI: 0.51 - 1.85). There were some differences in inclusion criteria and follow up time of the AHS cohort between these studies. In addition, in contrast to the most recent publication from the AHS cohort, Leon *et al*. (2019) did not control for cigarette smoking, alcohol consumption or family history of cancer, while they did control for animal production and for different pesticide active ingredients from those included in the AHS publication. Further, in the recent pooled re-analysis by Pahwa *et al*. (2019) including the two non-Hodgkin's Lymphoma case-control studies by McDuffie *et al*. (2001) and DeRoos *et al*. (2003) no associations with glyphosate-based herbicide exposure and diffuse large B-cell lymphoma was found. RAC notes that the strength of this study (Leon *et al*., 2019) was the large sample size and the cohort design, and the limitation was that pesticide use in the AGRICAN and CNAP cohorts was derived from self-reported history of crops cultivated combined with crop-exposure matrices with no direct information based on self-reported use of glyphosate-

based herbicides or any other pesticide assessed. Furthermore, the exposure assessment (ever/never) did not allow dose-response analyses. RAC notes that missing data in the AGRICAN cohort (crop, pesticide treatment task, period of production and period of pesticide treatment task) and in the AHS cohort (pesticides applied) were imputed as described in White *et al*. (2011) and Heltshe *et al*. (2012), respectively. There was no imputation needed for CNAP since exposure data were derived from compulsory agricultural censuses and the information collected from the farmers were complete.

Overall, the studies based on the AHS data do not provide clear evidence that glyphosate-based herbicide exposure is associated with cancer. The finding of a possible association of acute myeloid leukaemia in the most recent update by Andreotti *et al*. (2018) should be looked at carefully in future updates. However, RAC notes that a high number of cancer sites were analysed in the AHS cohort so there is the possibility of statistical findings by chance.

No statistically significant association between exposure to glyphosate-based herbicides and multiple myeloma was reported in the pooled case-control study where a subset of three North America Pooled Project studies (Iowa, Nebraska and Canada) were included (502 cases, 2504 controls) (Presutti *et al*., 2016, Pahwa *et al*., 2019). In the study, self-reported information on pesticide use, farming activities and demographic characteristics was collected, and the OR were calculated for "ever/never" exposure, years of exposure (less or more than three years) and cumulated lifetime days of exposure (less or more than six lifetime days of exposure) to glyphosate-based herbicides with and without exclusion of proxy respondents. The adjusted OR including proxy respondents was 1.29 (95% CI: 0.90 - 1.85) and excluding proxy respondents 1.07 (95% CI: 0.69 - 1.66). RAC notes that confounding factors such as exposure to other pesticides, chemicals, or radiation as well as occurrence of multiple myeloma in first degree relatives were not taken into account.

Kachuri *et al*. (2013) did not find any association between exposure to glyphosate-based herbicides and multiple myeloma in Canadian men with lifetime exposure to multiple pesticides (OR 1.1, 95% CI: 0.66 - 1.86). However, a borderline significant association was reported when considering > 2 days/year of glyphosate-based herbicide use (OR 2.11, 95% CI: 0.95 - 4.70). RAC notes that this was based on a low number of exposed cases (n=10). Pahwa *et al*. (2012) analysed the same Canadian men but used a slightly different analyses and reported no association between glyphosate-based herbicide exposure and multiple myeloma. RAC notes that the difference between to two studies were that Kachuri *et al*. (2013) excluded 10% of controls who did not have an age match, adjusted the ORs for smoking and provided a separate analysis for proxy respondents.

Statistically significant associations between exposure to glyphosate-based herbicide and non-Hodgkin's Lymphoma have been reported in case-control studies in the Swedish, Canadian and US populations. However, when adjustment for confounding factors was applied, the effects were no longer statistically significant in most studies. In the Swedish case-control study which included 910 cases of non-Hodgkin's Lymphoma and 1016 controls living in Sweden, 29 persons with non-Hodgkin's Lymphoma and 18 control persons reported exposure to glyphosate-based herbicides giving an initial odds ratio[1] (OR) 2.02, 95% CI: 1.10 - 3.71 (Eriksson *et al*., 2008), when adjusted for age, sex and year of diagnosis (cases) or enrolment (controls). When it was adjusted for co-exposure to agents other than glyphosate-based herbicides using multivariate

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[1] An odds ratio (OR) is a measure of association between an exposure and an outcome. The OR represents the odds that an outcome will occur given a particular exposure, compared to the odds of the outcome occurring in the absence of that exposure. Odds ratios are most commonly used to measure an association in case-control studies.

analysis, the adjusted OR was not statistically significant (OR 1.51, 95% CI: 0.77 - 2.94). Hardell *et al*. (2002) found a significant increase of non-Hodgkin's Lymphoma in a Swedish case-control study which included 515 cases and 1141 controls (8 exposed glyphosate-based herbicide cases and 8 exposed controls) when using univariate analysis with OR 3.04, 95% CI: 1.08 - 8.52, but it also became non-significant when applying a multivariate analysis (OR 1.85, 95% CI: 0.55 - 6.20). Adjustments were made for use of other pesticides in the multivariate analysis. In Canadian men, McDuffie *et al*. (2001) reported an adjusted OR for non-Hodgkin's Lymphoma of 1.20 (95% CI: 0.83 - 1.74), adjusted for age, province, and medical variables (but not use of other pesticides) in a case-control study including 517 cases and 1506 controls. The OR was significant for only cases with more than 2 days exposure per year, compared to those with less (OR 2.12, 95% CI: 1.20 - 3.73). In mid-western US the risk for non-Hodgkin's Lymphoma when exposed to glyphosate-based herbicides was found to be statistically significantly increased with 36 exposed cases of non-Hodgkin's Lymphoma and 61 controls with first grade logistic regression OR 2.1 (95% CI: 1.1 - 4.0) (De Roos *et al*., 2003). Adjustments were made for use of other pesticides. When second stage hierarchical regression was applied, the association was not statistically significant, with OR 1.6 (95% CI: 0.9 - 2.8). This was based on analyses of pooled data from three case-control studies (Cantor *et al*. 1992; Zahm *et al*., 1990; Hoar *et al*., 1986) from the NCI, including 622 cases/1245 controls, 201 cases/725 controls and 170 cases/948 controls, respectively. It was noted that a high number of proxy respondents were included in the study (40% for cases and 31% for controls). In analyses of multiple pesticides, there were 650 cases and 1933 controls following exclusion of subjects with missing data. In a French case-control study which included 244 cases and 436 controls, Orsi *et al*. (2009) did not find an increased risk (OR 1.0, 95% CI: 0.5 - 2.2, of 12 exposed cases and 24 exposed controls).

Proxy respondents were used in the pooled analysis of three case-control studies by De Roos *et al*. (2003), and in the case-control studies by Hardell *et al*. (2002) and McDuffie *et al*. (2001). Proxy respondents were not used by Eriksson *et al*. (2008) and Orsi *et al*. (2009).

In the hospital-based case-control study reported by Orsi *et al*. (2009), face-to-face interviews were conducted with the patients. All the other case-control studies described here were population-based, and self-administered questionnaires were distributed to cases and controls. The self-administered questionnaires were followed up by telephone interviews for clarification in the studies by Eriksson *et al*. (2008), Hardell *et al*. (2002), and McDuffie *et al*. (2001). The use of proxy respondents in some studies and questionnaire-based exposure information with the previously mentioned recollection related inaccuracy, both regarding exposure to glyphosate-based herbicides and exposure to other pesticides, indicate that effects of confounding and bias cannot be ruled out in those studies or in the meta risk estimates relying on those studies. This is the case even if efforts were made to minimise them.

Exposure-response trend was investigated by De Roos *et al*. (2003) as multiple pesticide use, and by Eriksson *et al*. (2008) as exposure for more or less than 10 days per year, and by McDuffie as days/year of exposure (mixing or applying pesticides). It needs to be mentioned that RAC considers multiple pesticide use not to be representative of an exposure-response analysis with regard to glyphosate-based herbicide exposure. RAC notes that while some indication of a dose-response relationship was observed in the Eriksson *et al*. (2008) and McDuffie *et al*. (2001) studies, these analyses did not adjust for confounding by exposure to other pesticides.

In the consultation (comment no. 31) the recent case-control study by Meloni *et al*. (2021) was included. This study was a part of the Italian "Gene-environment interactions in lymphoma etiology" (ItGxE) multicentre study from six Italian centres taking place between 2011 and 2017. The study included 867 lymphoma cases and 774 controls. The controls were either hospital controls recruited from other hospital departments (exclusion criteria were well defined) or random controls from the general population, depending on the centre. Detailed questionnaire

information on the duration, confidence (representing the degree of certainty about whether the study subject had actually been exposed), frequency (low frequency ≤ 5 days/year; medium frequency 5 - 10 days/year; high frequency ≥ 11 days/year), and intensity of exposure to glyphosate-based herbicides for each study subject was provided. Using unconditional regression analysis, the risk of major lymphoma (all subtypes), the non-Hodgkin's Lymphoma and B-cell lymphoma, Hodgkin's Lymphoma, and the major B-cell lymphoma subtypes, including diffuse large B-cell lymphoma, chronic lymphocytic leukaemia, follicular lymphoma, and multiple myeloma, associated with exposure to glyphosate-based herbicides and adjusted by age, gender, education, and study centre was modelled. The study did not assess the confounder effect of co-exposure to other pesticides. Among the participants only 36 participants (2.2%) were ever exposed to glyphosate-based herbicides. The results did not show an association with increased risk of lymphoma (any subtype), non-Hodgkin's Lymphoma, B-cell lymphoma or the major lymphoma subtypes. However, the risk of follicular lymphoma was increased in subjects classified as ever exposed to glyphosate-based herbicides with medium and high confidence (OR 7.1, 95% CI: 1.57 - 31.9) (3 cases), with medium-high cumulative exposure (OR 4.5, 95% CI: 0.82 - 24.1) (2 cases), with medium-high exposure intensity (OR 12.0, 95% CI: 2.95 - 49.0) (4 cases), and with exposure for 5 - 10 days or more per year (OR 6.0, 95% CI: 1.40 - 26.1) (3 cases). Due to the very few study subjects (n=36) with ever exposure to glyphosate-based herbicides, the study suffered from low statistical power. This increased the probability of chance findings with a very wide 95% CI (1.06 - 12.79) for follicular lymphoma. In the other case-report studies no follicular lymphoma were reported. Leon *et al*. (2019) did not find an increased risk of follicular lymphoma in association with ever exposure to glyphosate-based herbicides. Orsi *et al*. (2009) found a non-significant 40% excess risk of follicular lymphoma in ever exposed subjects (3 cases), however, the study suffered from a low prevalence of exposure.

Confounders and other obstacles to causal inference were described in the CLH dossier, such as:

− exposure to other constituents in glyphosate-based herbicide,
− exposure to other pesticides,
− use of questionnaires and interviews and,
− poor recollection of exposure to glyphosate-based herbicide, use of proxy respondents,
− no measurement of blood biomarkers,
− lack of power due to small number of cancer cases,
− changes over time in the definition of non-Hodgkin's Lymphoma,
− history of family cancer incidences.

RAC notes that 'confounding' in epidemiology refers to a situation where a factor other than the one to be assessed correlates both with exposure and outcome, e.g., a co-formulant in glyphosate-based formulations would be a confounder if it would be at the same time a risk factor for the outcome in question (cancer or more specifically non-Hodgkin's Lymphoma). Further, RAC notes that measured blood biomarkers would more securely indicate any correlation between exposure and non-Hodgkin's Lymphoma and that there are some biomonitoring data available, e.g., Curwin *et al*. (2007). In this study, urinary levels of glyphosate were not higher among children, mothers, and fathers living in a farm household compared to families in non-farm households in Iowa, US. In fact, the glyphosate levels were higher among the non-farm children than the farm children. Covariates such as amount of pesticide applied or playing in treated fields did not correlate with urinary levels. More recent biomonitoring studies are available and are in line with the previous levels (Connolly *et al*., 2018b; Conrad *et al*., 2017; McGuire *et al*., 2016; Sierra-Diaz *et al*., 2019; Trasande *et al*., 2020).

RAC notes that the co-formulant Polyethoxylated (POE)-tallowamine (CAS No 61791-26-2) was allowed to be used in glyphosate-based herbicides in Europe until 2016. Since then, '*Member States shall ensure that plant protection products containing glyphosate do not contain the co-*

formulant POE-tallowamine' (see Commission Implementing Regulation (EU) 2016/1313). According to the EFSA evaluation (2015), significant toxicity of POE-tallowamine has been observed for the endpoints for which data exist. However, no data are available regarding long-term toxicity and carcinogenicity of POE-tallowamine on the EFSA website since 2015.

RAC acknowledges that due to their nature, epidemiological studies are subject to a greater level of uncertainty compared to experimental studies, since exposure and other conditions are not controlled by the investigator. Consequently, bias, confounding factors, inaccuracies in exposure assessment etc. need to be minimised when designing and performing an epidemiology study. RAC notes that epidemiology is a highly relevant way to study effects in humans, as is also acknowledged by the CLP Regulation and guidance.

<u>Reviews, re-analyses and meta-analysis of non-Hodgkin's Lymphoma and multiple myeloma</u>

Reviews and re-assessments of the AHS data were conducted by: Sorahan (2015), Alavanja *et al*. (2013), Mink *et al*. (2012), Weichenthal *et al*. (2010) and Pahwa *et al*. (2019), Crump *et al*. (2019) and Weisenburger (2021).

In a study sponsored by Monsanto, Sorahan (2015) re-analysed the data for multiple myeloma reported by De Roos *et al*. (2005), and concluded that the risk given by De Roos (RR 2.6, 95% CI: 0.7 - 9.4) was due to an unrepresentative restricted dataset where subjects with missing data were excluded from the main analysis and that there was no convincing link between the glyphosate-based herbicide use and the risk of multiple myeloma. When using the full dataset and adjusting for a) age and gender, and b) lifestyle factors, the RR decreased to 1.12 (95% CI: 0.50 - 2.49) and 1.24 (95% CI: 0.52 - 2.94), respectively.

Alavanja *et al*. (2013) did not re-analyse data but compiled results from multiple epidemiological studies of the relationship between exposure to pesticides and the risk of cancer. They mentioned one positive study by Eriksson *et al*. (2008) and the association between glyphosate-based herbicide and non-Hodgkin's Lymphoma, but other negative studies are not mentioned.

In another study sponsored study by Monsanto, Mink *et al*. (2012) reviewed the quality 14 case-control studies to evaluate whether exposure to glyphosate-based herbicide was associated causally with risk of any type of cancer in humans. The case-control studies reporting on the relationship between exposure to glyphosate-based herbicides and risk of non-Hodgkin's Lymphoma were the following: Cantor *et al*. (1992), Nordstrom (1998), Hardell and Eriksson (1999), McDuffie *et al*. (2001), Hardell *et al*. (2002), De Roos *et al*. (2003), Lee *et al*. (2004a), Eriksson *et al*. (2008). Mink *et al*. (2012) stated that all of the studies were prone to bias, measurement error, and/or confounding, and concluded that with a cautious interpretation of the few positive associations reported in the literature, the epidemiological data considered together do not support a causal association between glyphosate-based herbicide exposure and cancer. No meta-analysis was performed as the authors did not consider it appropriate to calculate quantitative summary relative risk estimates across studies evaluating different site-specific cancers.

In a review of cancer incidence in 28 epidemiological studies of pesticide exposure and cancer incidence in the AHS cohort, Weichenthal *et al*. (2010) stated that glyphosate-based herbicides were not associated with non-Hodgkin's Lymphoma or any other cancer type in pesticide applicators. Exposure misclassification was mentioned as a concern. RAC notes that the recent update of the AHS cohort by Andreotti *et al*. (2018) was not included in this review.

A recent pooled re-analysis by Pahwa *et al*. (2019) included the two non-Hodgkin's Lymphoma case-control studies by McDuffie *et al*. (2001) and DeRoos *et al*. (2003). The re-analysis evaluated the associations of glyphosate-based herbicide use and non-Hodgkin's Lymphoma overall and by histological sub-types of non-Hodgkin's Lymphoma. Further, more control of

confounding factors was included, and the impact of excluding pesticide information provided by proxy respondents. The OR for non-Hodgkin's Lymphoma overall for ever using glyphosate-based herbicide was 1.4 (95% CI: 1.1 - 1.8). After adjustment for use of other pesticides, the OR was reduced to 1.1 (95% CI: 0.8 - 1.5). For diffuse large B-cell lymphoma the findings were similar. For other non-Hodgkin's Lymphoma subtypes, consistent patterns of no association between glyphosate-based herbicide exposure were reported. Exclusion of proxy respondents reduced ORs to a minor degree and all associations were not significant. A moderate association was reported that was borderline significant between non-Hodgkin's Lymphoma overall and diffuse large B-cell lymphoma with glyphosate-based herbicide exposure for > 2 days/year (OR 1.7, 95% CI: 1.02 - 2.94 and OR 2.14, 95% CI: 1.1 - 4.3, respectively). RAC notes that for non-Hodgkin's Lymphoma overall (n=30) and diffuse large B-cell lymphoma (n=14) a small number of cases were included with > 2 days of glyphosate-based herbicide use, respectively. Further, no trend in ORs were seen when cases with 0 to ≤ 2 days of glyphosate-based herbicide exposure were compared with cases with > 2 days of glyphosate-based herbicide exposure (for non-Hodgkin's Lymphoma overall and for diffuse large B-cell lymphoma both the P-value for trend was 0.2).

Crump *et al.* (2020, B.6.5.18.1) assessed the potential for recall bias in the main studies assessing the association between exposure to glyphosate-based herbicides and non-Hodgkin's Lymphoma. These included 5 case-control studies (Eriksson *et al*., 2008; Hardell *et al*., 2002; Mc Duffie *et al*., 2001; Orsi *et al*., 2009; De Roos *et al*., 2003) and two cohort studies (Andreotti *et al*., 2018; De Roos *et al*., 2005). The basis for the study by Crump *et al*. (2019) was that the percentage of odds ratios > 1 for non-glyphosate-based herbicide exposures should be approximately 50% if recall bias was not operative and the exposures did not cause non-Hodgkin's Lymphoma. In the assessment by Crump *et al*. (2019), it was shown that the percentages of ORs > 1 for non-glyphosate based herbicide exposures were 90% for Hardell *et al*. (2002), 90% for Eriksson *et al*. (2008), 93% for McDuffie *et al*. (2001), 76% for Orsi *et al*. (2009), and 53% for De Roos *et al*. (2003), showing percentages above 50% for four of the five case-control studies consistent with recall bias, that may also include selection bias in the studies by Hardell *et al*. (2002) and Eriksson *et al*. (2008) since these studies excluded some OR calculations for glyphosate based herbicide from the unexposed (to glyphosate) cases and controls who reported exposures to other pesticides. In contrast, in the most recent AHS cohort by Andreotti *et al*. (2018), only 48% of the RR calculated were > 1 and in the De Roos *et al*. (2005) 52%. These were percentages in the range expected with a true probability of 50%. Based on the high percentage of ORs above 1 it seems that recall bias may have played a factor in several case-control studies and has to be taken into account in the evaluation of these studies by RAC.

In the review by Weisenburger (2021), the scientific literature linking exposure to glyphosate and glyphosate-based-herbicides to the development of non-Hodgkin's Lymphoma was examined, with emphasis on new findings since the publication of the IARC Monograph 112 (2015). The review included both animal data, epidemiological data as well as mechanistic data. The literature was evaluated and related to the Bradford Hill criteria of causation. Weisenburger (2021) concluded that seven of the eight Bradford Hill criteria were fully or partly fulfilled. The author acknowledged that the results were not consistent, since cohorts of higher reliability (AHS cohort) did not confirm the postulated positive associations found by Weisenburger (2021) in the case-control studies (Mc Duffy *et al*., 2001; Hardell *et al*., 2002; De Roos *et al*., 2003; Eriksson and Hardell, 2008; Orsi *et al*., 2009). Furthermore, Weisenburger (2021) argued that negative result of the AHS cohort should not be used to negate the results of case-control studies, since lifetime years of exposure to glyphosate-based herbicides still remains low. RAC takes note of the publication and has reviewed the studies included in the publication taking it into account in a weight of evidence in the opinion.

In a meta-analysis, the risk estimates (OR or RR) from several studies are combined in a way that the statistical accuracy of the study (size of the study) and not the magnitude of the risk estimate defines their weight in the overall weighted meta-RR. Still the meta-analyses carry over any potential bias or confounding that might be in the risk estimates of those individual studies, e.g.: any effect that may come from recall bias or use of proxy respondents.

Systematic review and meta-analysis by Chang and Delzell (2016)

Chang and Delzell (2016) published a systematic review and meta-analysis, sponsored by Monsanto, on glyphosate-based herbicide exposure and risk of lymphohaematopoietic cancers. In the meta-analysis (i.a. on the following studies reporting on non-Hodgkin's Lymphoma and non-Hodgkin's Lymphoma subtypes: De Roos *et al.*, 2005 and 2003; Eriksson *et al.*, 2008; Hardell *et al.*, 2002; McDuffie *et al.*, 2001; Orsi *et al.*, 2009; Cocco *et al.*, 2013), they concluded that they found marginally significant positive meta-relative risks (meta-RRs) for the association between glyphosate-based herbicide use and risk of non-Hodgkin's Lymphoma (meta-RRs 1.3, 95% CI: 1.0 - 1.6) when using the most adjusted risk estimate from the studies. In a meta-analysis of the studies of Orsi *et al.* (2009), Sorahan (2015), Brown (1993), and Kachuri *et al.* (2013) there was a slight significant positive meta-RR for the association between glyphosate-based herbicide use and risk of multiple myeloma (meta-RR 1.4, 95% CI: 1.0 - 1.9). There were statistically null associations with Hodgkin's Lymphoma based on the studies of Orsi *et al.* (2009) and Karunanayake (2012) (meta-RR 1.1, 95% CI: 0.7 - 1.6) and leukaemia based on the studies of De Roos *et al.* (2005), Brown (1990), and Kaufman (2009) (meta-RR 1.0, 95% CI: 0.6 - 1.5). Even though there was a slight positive association between glyphosate-based herbicide use and non-Hodgkin's Lymphoma and multiple myeloma, the authors could not substantiate a causal relationship due to considerations in light of the Bradford Hill causality criteria. The results are presented in the figure below, reproduced from Figure 1 in Chang and Delzell (2016). The authors selected the newer studies while still covering all available data from older publications.

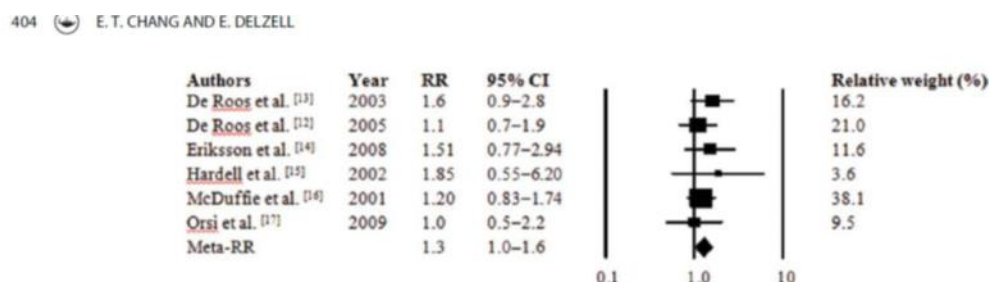**Figure:** *non-Hodgkin's Lymphoma analyses from Chang and Delzell (2016)*

Figure 1. Forest plots of relative risk (RR) estimates and 95% confidence intervals (CIs) for the association between glyphosate exposure and risk of non-Hodgkin lymphoma. Meta-RRs were identical in random-effects and fixed-effects models.

Chang and Delzell (2016) also analysed multiple myeloma, and came up with the following forest plots:

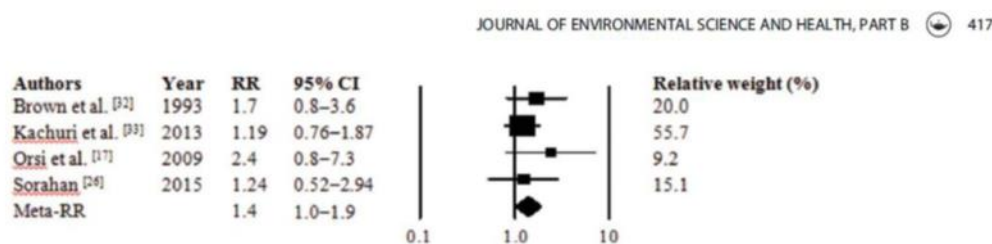**Figure:** *multiple myeloma analyses from Chang and Delzell (2016)*

Figure 2. Forest plots of relative risk (RR) estimates and 95% confidence intervals (CIs) for the association between glyphosate exposure and risk of multiple myeloma. Meta-RRs were identical in random-effects and fixed-effects models.

Systematic review and meta-analysis by Schinasi and Leon (2014)

A systematic review and meta-analysis for all studied populations was performed by the IARC scientists Schinasi and Leon (2014), who found a positive association between glyphosate-based herbicide use and non-Hodgkin's Lymphoma risk when the following studies were meta-analysed: McDuffie *et al*. (2001), Hardell *et al*. (2002), De Roos *et al*. (2003; 2005), Eriksson *et al*. (2008), Orsi *et al*. (2009). The meta-risk ratio estimate for glyphosate-based herbicide and non-Hodgkin's Lymphoma was 1.5, 95% CI: 1.1 - 2.0, and it was stronger (meta-RR 2.3, 95% CI: 1.4 - 4.0) in the studies diagnosed in the period 1975 - 1989 compared to more recent periods. The strongest meta-RR estimates were associated with subtypes of non-Hodgkin's Lymphoma. For B-cell lymphoma the meta-RR was 2.0 (95% CI: 1.1 - 3.6) based on only two studies (Cocco *et al*., 2013 and Eriksson *et al*., 2008), and identical to the result of Chang and Delzell (2016) based on the same studies. A possible causal relationship was not discussed by Schinasi and Leon (2014).

The IARC Monograph working group addressed the same studies as Schinasi and Leon (2014) but used the most fully adjusted risk estimates from the articles by Hardell *et al*. (2002), and Eriksson *et al*. (2008). The resulting meta-RR for glyphosate-based herbicide and non-Hodgkin's Lymphoma was 1.3 (95% CI: 1.03 - 1.65), i.e., the same as the meta-RR calculated by Chang and Delzell (2016, meta-RR 1.3, 95% CI: 1.0 - 1.6), based on the same studies.

The Epilymph study of B-cell lymphoma was a part of the meta-analyses of both Chang and Delzell (2016), and Schinasi and Leon (2014), who both concluded on a meta-risk ratio estimate of 2.0 (95% CI: 1.1 - 3.6) when the Epilymph study and Eriksson *et al*. (2008) were analysed.

Systematic review and meta-analysis by Zhang et al. (2019)

Zhang *et al*. (2019) published a meta-analysis for exposure to glyphosate-based herbicides and the risk of non-Hodgkin's Lymphoma. The study authors had an *a priori* hypothesis that higher and longer cumulative glyphosate-based herbicide exposure with the longest lag or latency period are likely to yield higher non-Hodgkin's Lymphoma risk estimates than lower and shorter exposures. The study included the recent update of the AHS cohort by Andreotti *et al*. (2018) or the AHS cohort by De Roos *et al*. (2005) together with five case-control studies also assessed in previous meta-analysis (De Roos *et al*. 2003; Eriksson *et al*. 2008, Hardell *et al*., 2002, Mc Duffy *et al*., 2001; Orsi *et al*., 2001). Zhang *et al*. (2019) included the highest exposure groups when available from each study (higher levels, higher duration and/or with sufficient lag and latency) and reported overall meta-relative risk (meta-RR) of non-Hodgkin's Lymphoma in glyphosate-based herbicide exposed individuals of 1.41 (95% CI: 1.13 - 1.75) when the AHS by Andreotti *et al*. (2018) was included and meta-RR for non-Hodgkin's Lymphoma of 1.45 (95% CI: 1.11 - 1.91) when the AHS by De Roos *et al*. (2005) was included (see the figure and table below). The study reported several sensitivity analyses to assess the impact of including or excluding studies (see the table below).

Figure: *Forest plot meta-analysis using AHS 2018 and AHS 2005 (from Zhang et al., 2019)*
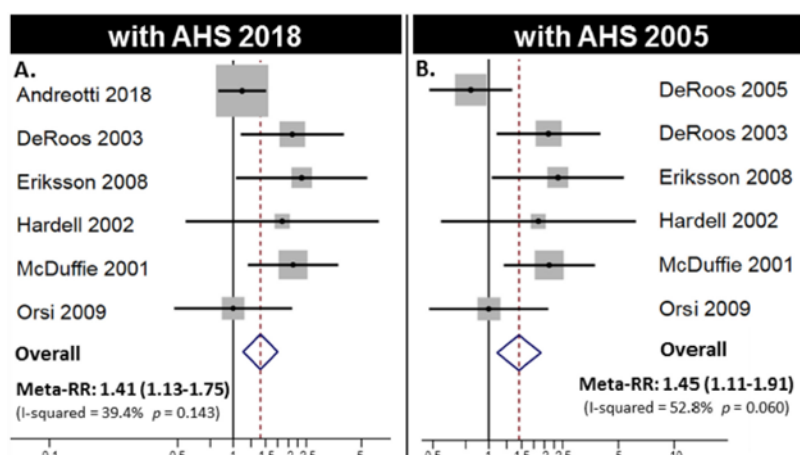
Table: *Results from Zhang et al. (2019)*

Analysis	Number of studies	Meta-RR (95% CI) for non-Hodgkin's Lymphoma
Highest cumulative* exposure		
• AHS (2018) • AHS (2005)	6 6	1.41 (95% CI: 1.13 - 1.75) 1.45 (95% CI: 1.11 - 1.91)
Longest exposure duration		
• AHS (2018) • AHS (2005)	6 6	1.41 (95% CI: 1.13 - 1.74) 1.56 (95% CI: 1.17 - 2.06)
Study design		
• Case-control** • Cohort (AHS 2018)	5 1	1.84 (95% CI: 1.33 - 2.55) 1.12 (95% CI: 0.83 - 1.51)

* Cumulative exposure includes duration and intensity.

** De Roos *et al.*, 2003; Eriksson *et al.*, 2008; Hardell *et al.*, 2002; Mc Duffy *et al.*, 2001; Orsi *et al.*, 2001.

Zhang *et al*. (2019) concluded that their findings were consistent with results reported from the other meta-analyses described above but show a higher risk for non-Hodgkin's Lymphoma because of their focus on the highest exposure groups. However, the authors noted that given the heterogeneity between the studies included (both case-control studies and cohort studies), the numerical risk estimates should be interpreted with caution. Furthermore, none of the available epidemiological studies/analysis captured the effects of the significant increased use of glyphosate-based herbicide beginning with the introduction of "green-burn-down" in the mid 2000s (Benbrook, 2016) as well as introduction of genetically modified glyphosate-resistant "Roundup-ready" crops in 1996.

Table: *Comparison of meta-analysis by Zhang et al. (2019) ("Current Meta-Analysis") with other published meta-analyses (from Zhang et al., 2019).*

Studies	Schinasi and Leon [25][a] RR (95% CI)	IARC [22] RR (95% CI)	Chang and Delzell [26][a,b] RR (95% CI)	Current Meta-Analysis	
				with AHS 2005 [19] RR (95% CI)	with AHS 2018 [24] RR (95% CI)
Andreotti *et al.* [24]	N/A	N/A	N/A	N/A	1.12 (0.83-1.51)
De Roos (2005) [19]	1.1 (0.7, 1.9)	1.1 (0.7, 1.9)	1.1 (0.7, 1.9)	0.8 (0.5, 1.4)	N/A
De Roos (2003) [15]	2.1 (1.1,4.0)	2.1 (1.1,4.0)	1.6 (0.9, 2.8)	2.1 (1.1,4.0)	2.1 (1.1,4.0)
Eriksson *et al.* [16]	2.0 (1.1,3.7)	1.51 (0.77, 2.94)	1.51 (0.77, 2.94)	2.36 (1.04, 5.37)	2.36 (1.04, 5.37)
Hardell *et al.* [17]	3.0 (1.1, 8.5)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)	1.85 (0.55, 6.20)
McDuffie *et al.* [42]	1.2 (0.8, 1.7)	1.20 (0.83, 1.74)	1.20 (0.83, 1.74)	2.12 (1.20, 3.73)	2.12 (1.20, 3.73)
Orsi *et al.* [18]	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)	1.0 (0.5, 2.2)
meta-RR (95% CI)	**1.45 (1.08, 1.95)[c]**	**1.30 (1.03, 1.64)**	**1.27 (1.01, 1.59)**	**1.45 (1.11, 1.91)**	**1.41 (1.13, 1.75)**

Abbreviations: CI, confidence interval; meta-RR, meta-relative risk; RR, relative risk;

[a] In their published reports, meta-RRs and their 95% confidence intervals were rounded to one digit right of the decimal point.

[b] Findings from Model 1, the primary analysis, are reported here.

[c] Random effects model.

Systematic review and meta-analysis by Kabat et al. (2021)

Kabat *et al*. (2021) was included in the consultation (comment no. 21) and evaluated the recent meta-analysis by Zhang *et al*. (2019) and performed a sensitivity analysis to determine how the definition of exposure and the choice of latency period affected the association risk for non-Hodgkin's Lymphoma. Kabat *et al*. (2021) also performed a meta-analysis of ever-exposure to glyphosate-based herbicide incorporating the most updated results from the four case–control studies (Eriksson *et al*., 2008; Hardell *et al*. 2002; Orsi *et al* 2001; Pahwa *et al*. 2019) and the AHS cohort study by Andreotti *et al*. (2018), see the figure below. Especially Kabat *et al*. (2021) highlighted the inconsistent definitions of exposure across the studies, the evidence of bias in case–control studies, the uncertainty about the latency period for non-Hodgkin's Lymphoma, and the selection of the highest of the available risk estimates from the AHS and from a pooled analysis of US case–control studies by De Roos *et al*. (2003) and performed a sensitivity analysis to investigate how these parameters affected the meta-estimates. Kabat *et al*. (2021) reported that the risk of non-Hodgkin's Lymphoma varied to a large extent depending on both the assumptions regarding exposure level and latency period, see table below. When using the highest reported exposure levels, evidence of an association between glyphosate-based herbicide and non-Hodgkin's Lymphoma was strongest when estimating a 20-year lag with a RR 1.41 (95% CI: 1.13 - 1.76) and a 15-year lag with a RR 1.25 (95% CI: 1.01 - 1.25). In the meta-analysis of ever-exposure with no lag period, the summary RR was 1.05 (95% CI: 0.87 - 1.28). Kabat *et al*. (2021) concluded that results of meta-analyses of glyphosate-based herbicide exposure and non-Hodgkin's Lymphoma risk depend on assumptions made about both exposure level and latency period. Kabat *et al*. (2021) further stated that one cannot say definitively that any particular meta-analysis is closest to the truth. They also pointed out that as cohort studies are considered less prone to bias and thus more reliable and evidence indicates bias in some case-control studies on glyphosate-based herbicides and non-Hodgkin's Lymphoma (see above Crump *et al*. 2020, B.6.5.18.1), any quantification of risk by combining data from the only cohort study (AHS) and the existing case-control studies should be interpreted with great caution.

Figure: *Forest plot including the 5 studies in the meta-analysis for ever-exposure to glyphosate-based herbicide (from Kabat et al. 2021).*
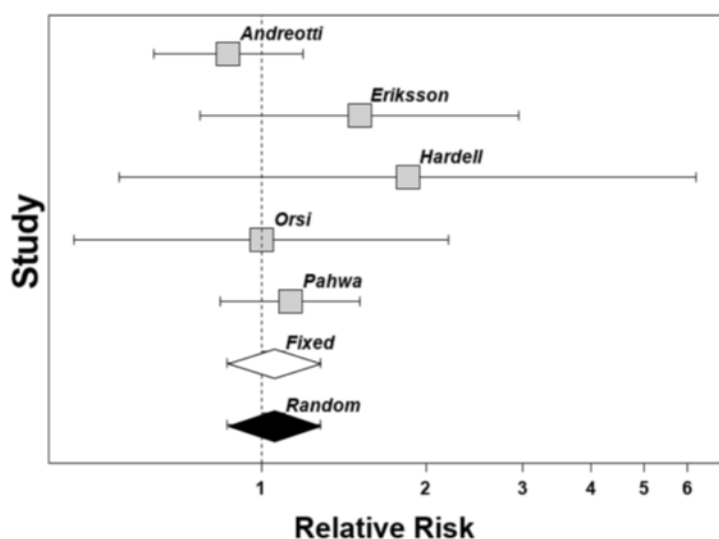
Table: *Individual study results for ever exposure to glyphosate-based herbicide and non-Hodgkin's Lymphoma risk, and summary relative risks for all studies, and from sensitivity analyses excluding one study at a time (from Kabat et al. 2021)*

	RR	LCL	UCL	Weight (%)
All studies				
1. Andreotti	0.87	0.64	1.19	38.5
2. Eriksson	1.51	0.77	2.95	8.48
3. Hardell	1.85	0.55	6.21	2.59
4. Orsi	1.00	0.45	2.20	6.13
5. Pahwa	1.13	0.84	1.52	44.3
Summary RR*	1.05	0.87	1.28	$Q^\dagger = 3.60$
Sensitivity analyses				
Exclude study 1	1.19	0.92	1.52	$Q = 1.30$
Exclude study 2	1.02	0.83	1.25	$Q = 2.38$
Exclude study 3	1.04	0.85	1.26	$Q = 2.75$
Exclude study 4	1.06	0.86	1.29	$Q = 3.58$
Exclude study 5	1.00	0.77	1.29	$Q = 3.20$

*Because heterogeneity was negligible ($I^2 = \tau^2 = 0$), the fixed effects and random effects summary RRs are identical

[†]If study estimates are homogeneous, Q has a chi-square distribution with $k - 1$ degrees of freedom, where $k =$ the number of studies

IARC

In 2015, IARC classified glyphosate as "probably carcinogenic to humans" (Group 2A), primarily based on animal studies. In their evaluation, the human data on carcinogenicity (primarily non-Hodgkin's Lymphoma) were described as limited.

US EPA Report of the cancer assessment review committee (CARC, 2015 and US EPA, 2020)

CARC (2015) concluded that the epidemiological evidence does not support a causal relationship between glyphosate-based herbicide exposure and solid tumours. Furthermore, for several types of non-solid tumours like Hodgkin's Lymphoma and multiple myeloma, CARC (2015) stated that there is no evidence to support a causal relationship. However, for non-Hodgkin's Lymphoma,

they decided that evidence from epidemiology is inconclusive for a causal associative relationship with glyphosate-based herbicide exposure. In a recent update, US EPA performed a review of the Zhang *et al*. (2019) and Leon *et al*. (2019) papers as part of the undergoing registration review in US (US EPA, 2020). The US EPA summarised from the Zhang *et al*. (2019) study that the *a priori* hypothesis that higher/longer exposures produce larger effect sizes in their analysis does not appear to be supported by the new AHS data from Andreotti *et al*. (2018) which is the largest, best-designed high-quality study examined. The US EPA summarised from the Leon *et al*. (2019) study that the combined three cohorts – one from France (AGRICAN), one from Norway (CNAP) and one from the US (AHS) did not find a statistically significant relationship between ever-exposure to glyphosate-based herbicide and non-Hodgkin's Lymphoma overall (HR 0.95, 95% CI: 0.77 - 1.18), n=1131 exposed cases). A somewhat elevated HR was found for one non-Hodgkin's Lymphoma subtype (diffuse large B-cell lymphoma) with a HR of 1.36, (95% CI: 1.00 - 1.85). The US EPA concluded that the additional information provided in Leon *et al.* (2019) does not impact the conclusions presented in the US EPA Revised Glyphosate Issue Paper which concluded that the strongest support based on the weight-of-evidence is for glyphosate being categorised as "not likely to be carcinogenic to humans".

Overall, available epidemiological case-control studies, reviews, re-analyses and meta-analyses show weak statistically significant associations between exposure to glyphosate-based herbicide and findings of cancer, especially non-Hodgkin's Lymphoma. This indicates a potential concern for human health. However, chance, bias and confounding factors could not be ruled out. A causal relationship with exposure to glyphosate-based herbicide can thus not be confirmed by RAC. More specifically, this is due to a number of factors – i.a. the weak associations which were only significant when certain statistical tests were applied, small studies with low number of exposed cases, the probability of recall bias for previous exposure (duration and dose) especially in the case-control studies, selection bias, the lack of biomonitoring data, frequently not adjusting for confounding factors such as co-exposure to other pesticides and risk estimates often getting lower when more comprehensive adjustment was applied, the presence of a toxic co-formulant (POE-tallowamine), and the changes in the definitions of non-Hodgkin's Lymphoma/other cancers over the years.

No association between exposure to glyphosate-based herbicides and incidences of non-Hodgkin's Lymphoma was observed in the only robust cohort study available.

The findings from the epidemiology studies are used in a weight of evidence approach together with the findings in animal studies. The comparison with the classification criteria is given in the section below.

Comparison with the CLP criteria

The database for the evaluation of glyphosate carcinogenicity is extensive and RAC bases its assessment on data from human epidemiological studies and a wide range of experimental animal carcinogenicity studies (7 rat and 5 mouse conventional cancer bioassays). The exposure route was oral in both the rat and the mouse studies, and the doses used were sufficiently high in all but one of the evaluated studies. There are no data suggesting that there are significant species differences and the studies performed and the tumour types evaluated are considered relevant to humans. The database includes studies of sufficient reliability and relevance to allow a robust evaluation following the requirements of the CLP Regulation.

Category 1A

Classification in Category 1A concerns substances known to have carcinogenic potential for humans and is largely based on human evidence.

Although available epidemiological case-control studies, reviews, re-analyses and meta-analyses show weak statistically significant associations between exposure to glyphosate-based herbicide and findings of cancer, especially non-Hodgkin's Lymphoma, chance, bias and confounding factors could not be ruled out. The AHS cohort study is considered by RAC as the most robust epidemiological study since it includes appropriate controls, a balanced assessment, and due consideration of bias or confounding factors. No association between exposure to glyphosate-based herbicide and non-Hodgkin's Lymphoma was found in the AHS cohort study. A causal relationship to cancer following exposure to glyphosate-based herbicide can thus not be confirmed by RAC.

Hence, classification of glyphosate in Category 1A is not justified. The detailed reasoning has been provided above.

Category 1B

Category 1B is for substances presumed to have carcinogenic potential for humans. Classification is largely based on animal evidence.

Following an overall evaluation of the human evidence and the tumour data from seven rat and five mouse bioassays it is concluded that there is not sufficient evidence for carcinogenicity and a classification of glyphosate in Category 1B is thus not warranted. The evaluation of strength of evidence and additional considerations including biological relevance of the tumour data is provided for each tumour type above. The main arguments are briefly summarised below.

Category 2

Category 2 substances are suspected human carcinogens. Classification is based on evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations. RAC notes the following in relation to glyphosate:

Epidemiological data:

> No association between exposure to glyphosate-based herbicide and non-Hodgkin's Lymphoma was found in the AHS cohort, which is the only prospective cohort study available. Weak positive associations have been observed in some case-control studies, and in meta-analyses of glyphosate-based herbicide exposure and non-Hodgkin's Lymphoma, as concluded in the meta-analyses by Chang and Delzell (2016), Schinasi and Leon (2014), Zhang *et al*. (2019), and also in IARC Monograph 112 (2015). However, Kabat *et al*. (2021) concluded that results of meta-analyses of glyphosate-based herbicide exposure and non-Hodgkin's Lymphoma risk depend on assumptions made about both exposure level and latency period. RAC notes that the increased risk of non-Hodgkin's Lymphoma observed in some case-control studies was not consistently observed in all case-control studies nor in the only cohort study available. For cancers other than non-Hodgkin's Lymphoma, there are less studies available and no consistent indication of an increased risk. In the AHS cohort an association between acute myeloid leukaemia and exposure to glyphosate was reported for the highest quartile of exposure when a 20-year lag period was taken into account, however, a low number of cases was found in this exposure group. RAC notes that this tumour type should be followed in future updates of the AHS. A causal relationship could not be established by RAC because chance, bias, and confounding factors could not be ruled out, and the evidence from epidemiological studies was considered insufficient to demonstrate carcinogenicity in humans.

Animal bioassays:

- There is insufficient evidence to support a classification in Category 2 based on the evaluation of seven rat studies. A significant increase in benign pancreatic tumours, was

observed in males in the low dose groups of two studies (CA 5.5/011, 1981; CA 5.5/010, 1990), but no apparent dose-response relationships were seen. No similar increase in tumour incidences was reported for female rats in these two studies and no similar indication of pancreatic tumours were observed in any of the five other long-term studies for either males or females. The same holds true for liver adenomas that were increased in two of seven rat studies (CA 5.5/10, 1990; CA 5.5/002, 2001) and thyroid C-cell adenomas that were increased only in the study CA 5.5/010 (1990). The incidences of liver adenomas were within, whereas the incidences of thyroid tumours were slightly above, the range of the historical control data. The conclusion is supported by the benign nature of the tumours with no suggestions of progression towards malignancy, a low strength of the evidence and a lack of consistency between sexes and across the many studies performed.

An increased trend of skin basal tumours was reported in the CA 5.5/004 (1997) study but not in the five other carcinogenicity studies in rats, nor in female rats and it is considered to be of equivocal relevance. Further, no clear effects on the skin were reported following systemic exposure to glyphosate in the repeated dose toxicity studies in animals.

The increased incidences of skin keratoacanthomas in male rats were either non-significant, borderline, or significant depending on the statistical method used. Skin keratoacanthomas were reported in male rats but not in female rats. The incidences exceeded the available HCD; however, it is noted that the HCD are very limited for the induction of skin keratoacanthomas in male rats. Furthermore, the increased incidences in skin keratoacanthomas were only observed at very high dose levels, which slightly exceeded the maximum recommend dose rate according to the OECD TG. It was also noted that skin keratoacanthoma is a benign tumour which is shown to be rather common in aged male rats (Zwicker *et al.*, 1992). Further, it was noted that no malignant squamous cell carcinomas were reported.

In one study (CA 5.5/001, 2009), the incidence of pituitary adenomas was increased in both males and females. This is a common tumour in rats and no similar increase was reported in the other rat bioassays.

– In the mouse, four tumour types were considered in detail. These were renal tubular tumours, haemangiosarcomas, haemangiomas and malignant lymphomas. An increase in renal tumours was reported in males in the high exposure group in three of the five studies. Increased incidences in haemangiosarcoma were reported in CD-1 males at the top dose in two studies, and an increased incidence of haemangioma was reported in female mice in two out of five studies. Further, an increased incidence of malignant lymphoma was reported in three carcinogenicity studies in CD-1 mice and one study in Swiss albino mice. The increases in tumour incidences were all non-significant in pairwise comparisons with control groups by the Fisher's exact test. However, several of the findings were significant when tested by the Cochran-Armitage trend test. RAC considered that the findings in the individual mouse studies were not by themselves strong enough to warrant classification. This is based mainly on an evaluation of statistical significance, biological relevance and consistency of the findings, including comparison with HCD and differences in findings between the sexes. Increased tumour incidences observed at doses above 4000 mg/kg bw/d were given less weight by RAC because the doses used were excessive and exceeded the MTD. Looking at the overall pattern of tumour incidences, RAC notes a tendency for increased incidences of malignant lymphomas in male mice in the high dose groups in four of the five studies available. However, the tumour incidences were highly variable, mostly within the available HCD incidences, and elevated tumour incidences were not supported by parallel increases in non-neoplastic lymph node lesions. Furthermore, the

findings were not consistent between sexes and were not supported by findings in the rat studies.

– Mode of action data: glyphosate is not reactive and no structural similarity to a substance(s) for which there is good evidence of carcinogenicity has been suggested. RAC does not find sufficient evidence to support a genotoxic mode of action for glyphosate, and RAC agreed on no classification for germ cell mutagenicity in both 2017 and in the preceding chapter on germ cell mutagenicity. Furthermore, the available data do not support non-genotoxic modes of action such as growth stimulation or tissue necrosis. However, there is some evidence that glyphosate may induce oxidative stress in some tissues. Oxidative stress is a recognised mechanism by which non-DNA reactive chemicals may induce oxidative DNA-lesions. If not repaired, such lesions may in turn cause increased mutations and CA (Smith *et al.*, 2016). ROS and oxidative damage to macromolecules, including DNA, also occurs in normal physiology and in several pathological conditions not associated with increased cancer risk. Immunosuppression is a recognised risk factor for non-Hodgkin's Lymphoma, but the data for glyphosate are regarded as insufficient for evaluation of this mechanism.

RAC concludes that based on the epidemiological data as well as the data from long-term studies in rats and mice, taking a weight of evidence approach, **no classification for carcinogenicity is warranted**.

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Adverse effects on sexual function and fertility

The DS noted that the reproductive toxicity potential of glyphosate was investigated in a large number of two-generation studies in rats, only six of which could be considered either fully valid or supplementary. These studies were summarised in table 57 of the CLH dossier, along with 1 supplementary one-generation study, 1 not acceptable one-generation study and 3 not acceptable three-generation studies. In addition, studies from the open literature were taken into account to evaluate intrinsic properties of glyphosate on reproductive tissues and organs in males and females (table 59, CLH dossier). The DS noted that no new standard reproductive toxicity studies (generational studies) were submitted for the assessment of fertility.

According to the DS, potentially relevant effects for classification included changes in sperm parameters, delayed sexual maturation, reduced litter size and lower fertility indices. Overall, the DS was of the opinion that these effects were not sufficient for a classification for sexual function and fertility.

Adverse effects on development

The CLH dossier summarised a large number of developmental toxicity and teratogenicity studies with glyphosate conducted in rats and rabbits (table 60, CLH dossier). The DS noted that no new standard developmental toxicity studies were submitted for the assessment of developmental toxicity.

The studies did not show any teratogenic potential in rats. At 3500 mg/kg bw/d, which resulted in maternal toxicity and in one study even mortality, post-implantation losses and both skeletal variations and retardations were observed (CA 5.6.2/003, 1991; CA 5.6.2/008, 1980). Ventricular septal defect noted in study CA 5.6.2/003 (1991) was also observed in one foetus each of the 300 and 1000 mg/kg bw/d groups in study CA 5.6.2/002 (1995) and a different

foetus (from a different litter) in the 300 mg/kg bw/d group displayed a right aortic arch. In the most recent study by CA 5.6.2/001 (1996), no effects were seen up to 1000 mg/kg bw/d i.e., the highest dose tested.

Overall, the rat studies revealed only slight developmental effects, which were confined to very high and maternally toxic dose levels.

In rabbits, developmental effects (which included dilated heart, visceral malformations, and ventricular septal defects as well as retarded ossification or supernumerary rib in some studies) and post-implantation losses were observed. The DS attributed these findings to glyphosate administration to the female rabbits. However, the DS also noted that these findings were confined to dose levels at which severe maternal toxicity was apparent.

The DS therefore concluded that based on animal studies no classification for developmental toxicity was warranted. Furthermore, the DS noted that no convincing evidence of reproductive or developmental effects of glyphosate could be derived from epidemiological studies or from *in vitro* or *in vivo* studies relevant to reproductive toxicity assessment.

Adverse effects on or via lactation

The DS summarised that in the generational studies reduced pup weight was observed in individual studies at limit dose level (1000 mg/kg bw/d) and above (table 63, CLH dossier). Delayed sexual maturation (preputial separation) was observed at limit test dose (1000 mg/kg bw/d), and distended caecum was observed at the very high dose of 2000 mg/kg bw/d. Further, available published literature did not provide conclusive evidence that glyphosate exposure negatively affects reproduction (table 65, CLH dossier). Overall, the available data did not show clear evidence of adverse effect in the offspring as a consequence of transfer in milk.

Comments received during consultation

Comment no. 82 - 106 submitted during the consultation were related to the hazard class reproductive toxicity. 19 comments supported the proposal for no classification. These comments were provided by Industry Trade Organisations, civil society NGO, individuals and one MSCA. Comment no. 102 from the GRG (Company-Manufacturer) supported the proposed no classification from the DS.

Comment no. 89 recommended considering two publications by Lesseur *et al.* (2021) indicating a link between glyphosate exposure, measured as glyphosate or the metabolite AMPA in urine, and preterm birth and effects on the length of the anogenital distance in new-borns. It was however noted that these publications were of limited relevance since it could not be excluded that the effects could also result from co-formulants in the glyphosate formulations. Furthermore, limited number of samples were included, and only one urine sample in the 2nd trimester was taken measuring urinary concentration of glyphosate and AMPA.

Comment no. 90 from an International NGO contained an in-depth evaluation of the studies for reproductive toxicity and indicated that some of the information were not properly reported by the applicants and that some publications were not included in the CLH dossier. They were of the opinion that the conclusion that glyphosate causes no adverse effects on reproduction is incorrect. The DS replied with a thorough evaluation of the detailed comments from the International NGO arguing for their evaluation of the studies. The DS also noted that the study by Mohammadi *et al.* (2021) and Lorenz *et al.* (2020) included in the comment should be considered in the assessment for a classification for effects on sexual function and fertility.

Comment no. 104 from an Academic institution stated that their analysis of the academic literature suggested that both glyphosate and its formulations may exhibit endocrine disrupting properties that impact reproductive function. However, they also note that the difference between

their conclusions and the conclusion from the DS is due to the inclusion or not of peer-reviewed academic studies that tested formulations of glyphosate which are not considered relevant by the DS. The DS compared the published studies included in the RAR with the studies assessed by the Academic institution and observed three studies which had not been considered for the current assessment. However, two of these, the Dallegrave *et al.* (2007) and Walsh *et al.* (2000) studies, which were included in the previous RAR (2015), were excluded since effects caused by co-formulants could not be ruled out. For the third study, Niemeyer *et al.* (2018), the relevance for the hazard and risk assessment of glyphosate was considered unclear since some studies results seemed to be based on simultaneous exposure to several pesticides and it was not possible to exclude that the effects could also result from co-formulants.

Assessment and comparison with the classification criteria

Adverse effects on sexual function and fertility

There are a large number of two-generation studies in rats available for glyphosate, however no new standard toxicity studies (generational studies) were identified or assessed by the DS. The DS assessed six of these studies for the purpose of classification (table below: modified from table 57 from the CLH dossier). There was also a one-generation range finding study in rats (CA 5.6.1/009, 1991) which was considered as supplementary due to the low number of animals, limited parameters investigated and lack of statistics. In addition, three three-generation studies in rats were included in the evaluation by the DS, although they were considered as not acceptable due to major reporting deficiencies. Two of the studies (CA 5.6.1/012, 1988; CA 5.6.1/014, 1981) used doses up to 30 mg/kg bw/d and did not show any treatment related effects. The third study (CA/5.6.1/013, 1985) used doses up to 5000 ppm (462 - 502 mg/kg bw/d), however due to reporting deficiencies and major deviations from OECD TG 416, the DS considered this study not to be acceptable. Furthermore, a one-generation study (CA 5.6.1/011, 1988) in rats with doses up to 10 mg/kg bw/d did not show any treatment related effects.

Table: *Reproductive (two-generation) studies with glyphosate in rats (based on table 57 from the CLH dossier). All studies were already included in the previous RAC opinion (CLH, 2016, RAC, 2017)*

Study, purity of glyphosate	Strain, route	Dose levels	NOAEL	LOAEL	Targets/ Main effects***
CA 5.6.1/001, CA 5.6.1/002, CA 5.6.1/003, 2007; 95.7% 2-generation reproduction study OECD TG 416 GLP Considered acceptable by the DS	Sprague-Dawley diet 28/sex/group	0, 1500, 5000, 15000 ppm (corresponding to approximately 0, 104, 351 and 1063 mg/kg bw/d in males and 0, 162, 530 and 1634 mg/kg bw/d for females)	Parental, offspring, reproductive: 5000 ppm (351 mg/kg bw/d)	Parental, offspring, reproductive: 15000 ppm (1063 - 1634 mg/kg bw/d)	Parental: liver, kidney wt↑ in females; Reproductive: homogenisation resistant spermatid count↓ (400 million/g in controls vs 309 million/g at 15000 ppm in F0); Off-spring: delay in preputial separation in F1 males; day 45.9 vs 43 days in control. Not associated with reduced bw. No effects on fertility in F1 generation.
CA 5.6.1/004, 2000; 97.6%	Wistar rat, derived AlpK:APfSD	0, 1000, 3000, 10000 ppm (corresponding to	Parental, toxicity: 10000 ppm		Parental, offspring: bw ↓ (F1 pups & F1-adults)

Study, purity of glyphosate	Strain, route	Dose levels	NOAEL	LOAEL	Targets/ Main effects***
2-generation reproduction study OECD TG 416 GLP Considered acceptable by the DS	Diet 26/sex/group	approximately 0, 99, 293 and 985 mg/kg bw/d for males and 0, 104, 323 and 1054 mg/kg bw/d for females)	(985/1054 mg/kg bw/d) Offspring toxicity: 3000 ppm (293/323 mg/kg bw/d) Reproductive: 10000 ppm (985/1054 mg/kg bw/d)		
CA 5.6.1/005, 1997; 94.61% 2-generation reproduction study OECD TG 416 GLP Considered acceptable by the DS	Sprague Dawley rat Diet 24/sex/group	0, 1200, 6000, 30000 ppm (corresponding to approximately 0, 84, 415 and 2151 mg/kg bw/d in males and 0, 97, 485 and 2532 mg/kg bw/d in females)	Parental, offspring, reproductive: 6000 ppm (417/485 mg/kg bw/d)		Parental: loose stool, bw ↓, caecum distention, organ wt changes; Offspring: bw↓, caecum distention
CA 5.6.1/006, 1993*; 96.8% 2-generation reproduction study OECD TG 416 GLP Considered supplementary by the DS	Wistar rats Diet 30/sex/group	0, 100, 1000, 10000 ppm (corresponding to approximately 0, 7.7, 77 and 770 mg/kg bw/d	Parental, offspring, reproductive: 10000 ppm (700 - 800 mg/kg bw/d)		No treatment-related effects observed at any dose level in either F0 or F1 adults or in F1 and F2 offspring
CA 5.6.1/007, CA 5.6.1/008, 1992; 99.2% 2-generation reproduction study OECD TG 416 GLP Considered acceptable by the DS	Sprague-Dawley rat Diet 28/sex/group	0, 1000, 3000, 10000 ppm (corresponding to approximately 0, 66, 197 and 668 mg/kg bw/d in males and 0, 75, 226 and 752 mg/kg bw/d in females)	Parental: 1000 ppm (197 mg/kg bw/d) Offspring, reproductive: 10000 ppm (668 mg/kg bw/d)	Parental: 3000 ppm (668 mg/kg bw/d) Offspring, reproductive: not established	Parental, offspring: bw↓, food & water ↑, histopathological changes in salivary glands in F0/F1 m/f
CA 5.6.1/009, 1991**; 98.6% One generation range finding study No guideline, not GLP. Considered supplementary by the DS	Sprague-Dawley rat Diet F0 females. 10 time-mated/group F1 generation: 10/sex/group	0, 3000, 10000, 30000 ppm (corresponding to approximately 0, 236 - 311, 799 - 1010 and 2515 - 2789 mg/kg bw/d for F0 females and 0, 355 - 402, 1191 - 1335 and 3918 - 4453 for F1 offspring).	The study is not suitable for NOAEL setting, acceptable as dose range finding only due to low number of animals, limited parameters investigated and no statistics.		No adverse effects on reproduction

Study, purity of glyphosate	Strain, route	Dose levels	NOAEL	LOAEL	Targets/ Main effects***
CA 5.6.1/010, 1990; 97.67% 2-generation reproduction study No guideline, similar to OECD TG 416 GLP Considered acceptable by the DS	Sprague-Dawley rat Diet 30/sex/group	0, 2000, 10000, 30000 ppm (corresponding to approximately 0, 152, 760 and 2280 mg/kg bw/d)	Parental, offspring, reproductive: 10000 ppm (720 - 760 mg/kg bw/d for males and 777 - 804 mg/kg bw/d for females)	Parental, offspring, reproductive: 30000 ppm (~2000 mg/kg bw/d)	Parental: bw gain↓, soft stool Reproductive: litter size ↓ (equivocal); Offspring: bw gain↓

* Supplementary study since dose levels might have been too low and no effects were seen at all.

** Supplementary range-finding one generation study to study CA 5.6.1/007 (1992), CA 5.6.1/008 (1992).

*** "Main effects" were statistically significant if body weight and organ weights or reproductive parameters (apart from reduced litter size in the study CA 5.6.1/010, 2010) were affected.

RAC reviewed each of these studies and found most of them to be acceptable for hazard classification in a weight of evidence assessment.

The study CA 5.6.1/001-003 (2007) was considered as acceptable by the DS. In this study, a reduction in homogenisation resistant spermatid count (399.9 million/gram in controls vs 309.0 million/gram at 15000 ppm corresponding to ~1000 mg/kg bw/d) was seen in the F0 generation. However, this was not reported in the F1 generation. A statistically significant delay in sexual maturation, seen as delayed preputial separation in F1 male pups, was also observed at dose levels of 15000 ppm. Preputial separation occurred after 45.9 days on average, compared to 43 days in the control group. This was not considered to be related to changes in F1 male bodyweight since the body weight was statistically significantly increased in the males with delayed preputial separation (body weight in controls 210 g compared to 230 g at 15000 ppm). The delayed onset of sexual maturation had no impact on subsequent reproductive performance. There were no treatment related effects on mating performance, fertility, and gestation length in F0 and F1 generations at doses of up to 1063 mg/kg bw/d in males and 1634 mg/kg bw/d for females. Further, no differences in litter size and viability were seen. The only systemic toxicity reported was a statistically significant increase in female liver and kidney weight (absolute and relative) in the high dose group in the F0 generation and in the liver weight (absolute and relative) in the F1 generation.

The study by Dai *et al.* (2016) was also assessed and considered by the DS to be reliable with restrictions. This study investigated effects of glyphosate on reproductive organs in male rats. The dose levels of glyphosate used were 0, 5, 50 or 500 mg/kg bw/d for 5 weeks with 8 rats/group. The only effects reported were a dose-related statistically significant reduction in seminal vesicle gland and coagulating gland weights (0.42, 0.37, 0.34, or 0.31 g in the 0, 5, 50 or 500 mg/kg bw/d dose group, respectively). Total sperm count was reduced in the high dose group, but without any clear dose-response relationship. No statistically significant changes were reported in the serum levels of testosterone, oestradiol, or progesterone, however there was a trend towards decreased serum concentrations with dose for testosterone and progesterone. In the other two-generation studies, no significant effects were reported on sperm quality or male reproductive organs at doses up to 2000 mg/kg bw/d.

The study CA 5.6.1/004 (2000) was considered acceptable by the DS. In this study, doses up to 970 mg/kg bw/d did not reveal any effects on mating performance, fertility, gestation, and litter size in the F0 and F1 generations. Sperm assessment did not reveal any effects in either

generation. No effects on pup body weight were reported at birth in the F1 and F2 generations. However, a reduction in body weight of F1A pups of 10% was observed in the high dose group (10000 ppm) resulting in a subsequent reduction of 5% in body weight of the selected F1 parent males for the duration of the mating period. In the F2 offspring no changes in body weight were reported. No effects on sexual maturation were reported in F1 males and females.

The study CA 5.6.1/005 (1997) was considered acceptable by the DS. In this study, doses up to 2000 mg/kg bw/d did not reveal any effects on mating performance, fertility, and litter size in F0 and F1 generations. The fertility index (%) was reduced in F1 females, but not statistically significantly (95.8, 95.8, 87.5 or 79.2% in the control, 1200, 6000 or 30000 ppm doses corresponding to 91.7/104.8, 458/530 or 2411/2760 mg/kg bw/d for males/females, respectively). No changes in the fertility index were reported in F0 females. Sperm assessment did not reveal any effects in any of the generations. General toxicity was reported in the F1 and F2 generations as loose stool and caecum distension in males and females and a decrease in male body weight in the high dose group. In the F1 and F2 offspring a statistically significant decrease in body weight from PND14 and a significant increase in caecum distension was reported in the high dose group. Effects on sexual maturation were not assessed in this study.

The study CA 5.6.1/006 (1993) was considered supplementary by the DS and showed no treatment-related effects at doses up to 10000 ppm (700 - 800 mg/kg bw/d) for two successive generations. It is however noted that the study is limited due to lack of any effects up to the highest dose tested, no sperm analysis, no investigation of sexual development in offspring and limited histopathology.

The study CA 5.6.1/007-008 (1992) was considered acceptable. In this study, rats were fed diets containing up to 10000 ppm (668/771 mg/kg bw/d F0/F1 males or 752/841 mg/kg bw/d F0/F1 females). Findings in parental animals consisted of increased water consumption (17%) in F1 females at 10000 ppm, increased food intake (3%) in F1 females at 10000 ppm during the latter stage of the first pre-mating period), reduced mean body weight (1 - 7%) in F1 males at 10000 ppm, and histopathological findings in salivary glands (parotid and submaxillary glands) in parental animals at ≥ 3000 ppm (197/230 mg/kg bw/d F0/F1 males or 226/245 mg/kg bw/d F0/F1 females), manifested as minimal hypertrophy of acinar cells with prominent granular cytoplasm. The findings in the parotid gland were observed in male and female animals of both generations at 3000 ppm (F0 males 3/28, F0 females 5/28, F1 males 4/23, F1 females 4/24) and at 10000 ppm (F0 males 12/26, F0 females 17/28, F1 males 11/23, F1 females 9/23). The findings in the submaxillary gland were observed in F0 females at 3000 ppm (F0 females 4/28), and in F0 and F1 females at 10000 ppm (F0 females 14/28, F1 females 3/23). Trend analysis conducted by DS showed that the dose-related increase was statistically significant. No treatment-related effects were observed in the offspring.

The study CA 5.6.1/009 (1991) was considered supplementary since few animals were used, limited parameters were investigated, no statistical analyses were conducted. The one-generation reproductive toxicity study is a preliminary assessment for a subsequent two-generation reproductive toxicity study. Groups of 10 time-mated Sprague-Dawley rats received daily dietary doses of 0, 3000, 10000 and 30000 ppm glyphosate (range intake per group 236 - 311, 799 - 1010 or 2515 - 2789 mg/kg bw/d) from GD3 through gestation to termination at the end of lactation. All females were allowed to litter and rear their young to weaning, then 10 male and 10 female offspring per group were selected and reared on their respective diets to six weeks of age. No adverse effects on reproduction parameters nor on survival of pups through weaning were observed in this study.

The study CA 5.6.1/010 (1990) was considered acceptable by the DS. This study showed a rather equivocal reduction in litter size at dose levels exceeding 2000 mg/kg bw/d. In the two litters produced by the F0 generation, a non-significant reduction of litter size by up to 10% was

observed. This effect was less pronounced in the F1 generation. A reduction in litter size was not confirmed in the study CA 5.6.1/005 (1997), where the same dietary concentrations of glyphosate were tested.

Human data

The DS did not include human data in their evaluation of effects on sexual function and fertility. However, the DS noted that several epidemiological studies investigating a possible impact of glyphosate exposure on fertility are available. The parameters included in the studies are fecundity, miscarriage, pre-term delivery, gestational diabetes mellitus, birth weights, congenital malformations, neural tube defects and the occurrence of attention-deficit disorder / attention-deficit hyperactive disorder (ADD/ADHD) in children. However, due to uncertainties regarding the exposure to glyphosate-based herbicides, simultaneous exposure to other pesticides, the data are not considered to be sufficient to establish a clear link between exposure to glyphosate and effects on reproductive toxicity.

During the consultation, two recent studies, Lesseur *et al.* (2021a) and Lesseur *et al.* (2021b) were submitted. Lesseur *et al.* (2021a) was a pilot study in 94 mother-infant pairs (45 female and 49 male) from The Infant Development and the Environment Study (TIDES). For each infant, two anogenital distance (AGD) measurements were collected after birth; the anopenile (AGD-AP) and anoscrotal (AGD-AS) distances for males, and anoclitoral (AGD-AC) and anofourchette distances (AGD-AF) for females. In female infants, maternal urinary glyphosate above the median was associated with longer AGD-AC ($\beta=1.48$, 95% CI: -0.01 - 3.0, p=0.05), but this was not significant after covariate adjustment. Increased AMPA was associated with longer AGD-AF ($\beta=1.96$, 95% CI: 0.44 - 3.5, p=0.01) after adjusting for infant size and age at AGD examination. No associations were detected in male offspring.

Lesseur *et. al.* (2021b) investigated associations between prenatal glyphosate exposure and length of gestation in TIDES. A shortened gestational length was associated with maternal glyphosate (HR: 1.31, 95% CI: 1.00 - 1.71) and AMPA (HR: 1.32, 95% CI: 1.00 - 1.73) only among spontaneous deliveries. RAC notes the studies indicate some concern however, the effects reported are not considered relevant for the classification for reproductive toxicity. In addition, it is unclear if the effects could be related to an exposure to the co-formulants. Limitations in the studies included small sample size, few pre-term deliveries and only one sample in the 2nd trimester was taken measuring urinary concentration of glyphosate and AMPA.

Other studies from the open literature

Male reproductive system

Effects on the male reproductive system were investigated in two *in vitro* and six *in vivo* studies following different treatment protocols. The study by Dai *et al.* (2016) was included in the assessment by RAC from 2017, however the other studies mentioned below were not assessed at that time.

Gorga *et al.* (2020) assessed *in vitro* effects of glyphosate on Sertoli cell physiology and reported no effects on Sertoli cell metabolism after 48-hour exposure of Sertoli cell culture (from 20-d old Sprague-Dawley rats) to 10 and 100 ppm of glyphosate. The study also evaluated the effects on glyphosate on blood-testis barrier function by measuring Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly (claudin11, occludin and ZO-1) and on testosterone regulation of blood-testis barrier integrity. *In vitro* exposure of Sertoli cell to glyphosate at 100 ppm altered Sertoli cell junction barrier permeability and decreased testosterone stimulated TER. Furthermore, a redistribution of claudin11 was observed. The study is considered reliable with restrictions as the

test material identity, in particular purity, was not specified; only 1 or 2 concentration levels were tested for the different parameters; and a positive control was lacking.

Forgacs *et al.* (2012) studied the production of testosterone *in vitro* in mouse Leydig cells with exposure to 600 μM glyphosate and found no effects on the induction of testosterone production in Leydig cells, or with exposure to 300 μM glyphosate by using recombinant human chorionic gonadotropin induction of testosterone. The DS assessed the study to be reliable with restrictions because the test substance was not characterised and the results of only one concentration were reported, and RAC supports this assessment.

Dai *et al.* (2016) observed a significant decrease in absolute (but not relative) weight of the seminal vesicle and coagulating gland in Sprague Dawley rats (8/dose group) after 5 weeks exposure to ≥ 50 mg/kg bw/d of isopropylamine salt of glyphosate. The weight decrease of the seminal vesicle gland and coagulating gland was 22% and 30% at the mid and high dose levels of 50 mg/kg bw/d and 500 mg/kg bw/d, respectively, but this effect was only accompanied by decreased sperm count at the highest dose level. The total sperm count was significantly decreased at 500 mg/kg bw/d. In addition, there was a dose-depended trend towards decreased serum concentrations of testosterone and progesterone. Testicular, epididymal and seminal vesicle gland histology showed no significant differences compared to controls. It could be noted that reduced body weight was observed at mid (10%) and high dose level (9%), although not statistically significant. The study is not according to OECD test guideline and not GLP compliant. RAC notes that limited parameters were investigated, low number of animals were used, and no details about clinical signs were reported.

Pham *et al.* (2019) reported sperm depleted seminiferous tubule in 35 days old Swiss mice (84% decrease in spermatozoa) exposed to 5 mg glyphosate/kg bw/d (purity 99.2%) in drinking water from embryonic day 10.5 to 20 days post-partum (5 male mice derived from 3-4 litters/group exposed to 0, 0.5, 5 or 50 mg/kg bw/d glyphosate). No similar effect was observed in the same study in mice sacrificed at later time points (8 months). Further, no dose-response was observed since the effect was only seen in the mid dose group. The study also reported a three times lower serum testosterone level compared to the control group in 35-d old male mice at 0.5 and 50 mg/kg bw/d, however, with no decreased in the mid dose group. However, no effects on testosterone levels were reported in 8-month-old mice. The study is not according to OECD test guideline and not GLP compliant. RAC notes the limitation of this study because of small group size, limited description of the study conditions and the results.

Johansson *et al.* (2018) observed no effects on the testis parameters investigated in 4-week-old SD rats (10/group) following exposure up to 0, 2.5 or 25 mg/kg bw/d glyphosate (purity ≥ 96%) over 2 weeks. The parameters investigated in the study were: intra-testicular testosterone levels, expression of key marker genes in the testes, testis histopathology, protein expression analysis and apoptotic activity. The study was not conducted according to OECD test guideline and not GLP compliant. RAC notes that the exposure duration was short (2 weeks only), endpoints were limited (for e.g., testes were not weighed) and only 2 dose levels of glyphosate were tested.

Manservisi *et al.* (2019) performed a pilot study in Sprague-Dawley rats for an extended-one generation study (OECD TG 443). In the pilot study, no effects on sperm parameters (number of mature spermatids in the testis, daily sperm production, number and sperm transit time through caput/corpus and cauda epididymis and morphology) were reported in SD-rats (10 males/group) exposed to glyphosate (purity > 99.5%) at 1.75 mg/kg bw/d during gestation and for additionally 13 weeks. RAC notes the low dose of glyphosate tested in the study.

During the consultation (comment no. 90) the study by Mohammadi *et al.* (2021) was included. Mohammadi *et al.* (2021) conducted a systematic review and meta-analysis on studies where alterations of sexual hormones including testosterone, luteinising hormone (LH), follicle-

stimulating hormone (FSH), and oestradiol in rats were studied. They screened 284 articles, of which eight were eligible for the meta-analysis [3 from Brazil (Dallegrave *et al.,* 2007; Romano *et al.,* 2012; Romano *et al.,* 2010), 2 from Nigeria (Abarikwu *et al.,* 2015; Owagboriaye *et al.,* 2017), 1 from China (Dai *et al.* 2016), 1 from Iran (Razi *et al.,* 2012), and 1 from Italy (Manservisi *et al.*, 2019)]. Weighted mean difference (WMD) with 95% CI: was applied for estimating the effect of glyphosate or glyphosate-based herbicide exposure on the sex hormone levels. The results of the meta-analysis showed an effect of glyphosate exposure on decreasing levels of testosterone (7 studies, WMD=-1.48 ng/mL, 95% CI: 0.61 - 2.34; p=0.001) and LH (3 studies, WMD=-2.03 mIu/mL, 95% CI: 0.71 - 3.34; p=0.003). No significant changes in FSH and oestradiol levels were found. RAC notes that in five of the eight studies the exposure was only to glyphosate-based herbicide, one study included exposure to both glyphosate and glyphosate-based herbicides, and the impact of co-formulant exposure was not assessed, limiting the relevance of this study for the assessment for a classification for effects on sexual function and fertility. RAC also notes that this study has to be considered along with the results of the studies performed in accordance with GLP and acceptable test guidelines.

During the RAC 60 meeting, the studies by Liu *et al.* (2022a; 2022b) were raised by one NGO. Liu *et al.* studied the effects of glyphosate on sperm quality and blood-testis barrier in Sprague-Dawley male rats. The rats were exposed to 0, 2 or 50 mg glyphosate/kg bw/d (18/group divided into 3 replicates/group, 6 animals/replicate) for 8 weeks (Liu *et al.*, 2022a) or 4 months (Liu *et al.,* 2022b). Effects on the blood-testis barrier was assessed in 1 rat/replicate (measured with TEM by using biotin as an indicator), unilateral testis histopathology in 2 rats/replicate and epididymis histopathology and sperm parameters in 3 rats/replicate. Results Liu *et al.* (2022a): in glyphosate exposed rats, histopathological changes evident as irregular arranged seminiferous tubules with intraepithelial vacuolisation were reported. In addition, the blood-testis barrier integrity was affected in the high dose group where biotin was shown to permeate into the seminiferous tubules (only showed by images in the publication, with no possibility for a quantitative assessment). Further, glyphosate exposure induced a significant effect on sperm motility in the high dose group (% motility: 68.83 ± 6.31, 66.83 ± 8.86 or 59.33 ± 6.75 in the control, 2 or 50 mg/kg bw/d dose groups, respectively), however, with no effect on the sperm concentration (evaluate by CASA). A statistically significant increase in sperm deformity was also reported (1.7%, 2.1% or 5% in the control, 2 or 50 mg/kg bw/d dose groups, respectively). However, limited changes were reported in the expression of marker genes involved in spermatogenesis. Mechanistically, the changes in glyphosate-exposed testis were accompanied by the increased interleukin (IL)-17A production, probably due to gut-microbes-derived Th17 cell migration. Furthermore, activation of IL-17A signalling triggered testicular oxidative damage. Results Liu *et al.* (2022b): a slight, although statistically significant decrease in sperm quality (e.g., motility and velocity parameters) and reduced sperm quantity was shown in glyphosate low and/or high exposure groups (results only shown in figures). In addition, glyphosate was shown to decrease blood-testis barrier integrity and alter blood-testis barrier ultrastructure. Further, testis histopathological observation showed irregular arranged spermatogenic cells and intraepithelial vacuolation. Testicular analyses showed a reduction of tight junction and gap junction related genes and proteins. These findings were further elaborated in the *in vitro* Sertoli cell experiment showing that glyphosate-induced ROS contributed to the downregulation of blood-testis barrier-related proteins in primary Sertoli cells cultures exposed to 10 µM glyphosate. RAC notes that no positive control was included. Glyphosate residues were detected by GC-MS/MS in serum (0.035 ± 0.010 or 0.146 ± 0.023 µg/mL, at 2 or 50 mg/kg bw/d, respectively) and testis (0.002 ± 0.001 or 0.016 ± 0.006 µg/g at 2 or 50 mg/kg bw/d, respectively). RAC notes that the results were not reported quantitatively in the studies, as well as the low number of animals used in the studies. Furthermore, the focus on mechanistical aspects related to effects on the testis in rats following exposure to glyphosate. RAC also notes that these studies have to

be considered along with the results of the studies performed in accordance with GLP and acceptable test guidelines.

Overall, none of the published data suggest that the male reproduction system was adversely affected by glyphosate exposure. One *in vitro* and one *in vivo* study indicated some effect on the blood-testis barrier, however, limited animals were included in the study as well as no quantitative measurements of the effect. The study by Dai *et al.* (2016) reported reduced sperm counts at 500 mg/kg bw/d. However, more recent studies in rats and mice do not indicate an adverse effect on the male reproductive organs, however, noting that lower doses of glyphosate were used in these studies. Further, in the regulatory study CA 5.6.1/001-003 (2007) a significant decrease in homogenisation-resistant spermatid count in F0 males was observed at approximately 1000 mg/kg bw/d. The study by Dai *et al.* (2016) was also included in the assessment by RAC in 2017 and the study is considered by the DS to be reliable with restrictions due to few animals used and limited parameters investigated. RAC is of the opinion that the review of the available studies published before and after the previous evaluation in 2017 is not sufficient in a weight of evidence assessment to conclude on a classification for adverse effects on sexual function and fertility.

Female reproductive system

Effects on maturation of oocytes was investigated in three *in vitro* studies.

In the non-guideline non-GLP study by Zhang *et al.* (2019), it was found that treatment with 200 or 500 µM glyphosate significantly decreased germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE) indicating effects on development oocytes in Kunming mice. Further, at 500 µM glyphosate an increase in the mRNA expression of sod3, gpx and cat genes was observed, suggesting enhanced ROS formation. Further misaligned chromosomes, abnormal spindle morphology and reduced p-MAPK protein levels in oocytes were observed. The mitochondrial membrane potential was lowered suggesting interference with the mitochondrial function of the oocytes. The expression of Bcl-2 protein decreased, while that of Bax protein increased, suggesting induced early apoptosis in oocytes. The mRNA expression of autophagy related genes (Ic3, atg14 and mtor) and expression of autophagy-related proteins (LC3 and Atg12) was increased suggesting induced autophagy in oocytes. The DS noted that the study was considered reliable with restrictions due to uncertainty regarding purity and source of the test substance, no cytotoxicity testing and lack of positive control. It was further noted that only GVBD and PBE was tested with several dose levels up to 500 µM glyphosate. The rest of the experiment was only performed at 500 µM glyphosate. RAC notes these limitations of the study.

Perego *et al.* (2017) investigated effects of glyphosate on ovarian cell proliferation, steroid production and gene expression using bovine granulosa cells (GC) and theca cells (TC) in *in vitro* models in a non-guideline non-GLP study (0, 0.5 or 5 µg glyphosate/mL). A slight, non-dose-related alteration in bovine GC proliferation and oestradiol production was observed at 5 µg/mL. No effects were reported on progesterone production in GC. Further, at the same concentration glyphosate did not show any effect on TC proliferation and steroidogenesis. Higher concentrations were also tested (0.01 and 0.3 mg/mL), without any effect. The isolated occurrence of the observed effects without any dose-response relationship, questions the biological significance of the findings. Further, the DS noted that the study was considered reliable with restrictions since the glyphosate tested was not sufficiently characterised, no positive controls were included, and the tests were conducted with only one or two test concentrations of glyphosate. RAC notes these limitations of the study.

Yahfoufi *et al.* (2020) investigated the effects of glyphosate (0 - 300 µM) on metaphase II mouse oocyte quality and embryo damage to obtain insight on its mechanisms of cellular action and the tolerance of oocytes and embryos towards glyphosate. The study indicates that glyphosate

causes disruption of the microtubule organising centre and chromosomal disorganisation in the oocytes in a dose dependent manner and in concentrations in the range of those found in human blood following accidental acute exposure. In addition, interference with intracellular zinc bioavailability and ROS accumulation were observed in the mouse oocytes. Further, in embryos zinc depletion and accumulation of ROS were also observed in a dose-related manner. The study is considered as supplementary data and is reliable with restrictions.

Overall, the effects reported in these three *in vitro* studies are considered by RAC to be of limited *in vivo* relevance and are not considered to indicate clear evidence of adverse effects on female fertility. Furthermore, in the guidance compliant generation studies no effects on female fertility parameters were reported. RAC notes that these studies were not included in the previous assessment by RAC from 2017.

Further in vivo studies investigating reproductive and developmental toxicity

In the pilot study by Manservisi *et al.* (2019) Sprague-Dawley rats (8/group) were exposed to 1.75 mg/kg bw/d of glyphosate (> 99.5% pure) in drinking water. The dose was equivalent to the US Acceptable Daily Intake. The F0 females were exposed from GD6 to the end of lactation, while the F1 animals continued to be exposed after weaning for an additional 6 or 13 weeks. No effects on reproductive parameters were observed after exposure to glyphosate. It was however noted that a statistically significantly increase in the AGD in males on PND4 (4.26 in exposed males vs 4.02 in controls) was reported. However, there were no statistically significant changes in male pup body weight on PND1 (6.8 ± 0.5 and 7.1 ± 0.2 in the control group and exposed group, respectively). In addition, a statistically significantly increased plasma level of TSH was reported in male animals exposed to 1.75 mg glyphosate/kg bw/d in the 6-week cohort (8.17 ng TSH/mL in exposed animals vs 4.23 ng TSH/mL in controls) but not in the cohort exposed for 13 weeks. This study was not included in the previous assessment by RAC from 2017. RAC notes that the study was not performed according to a guideline nor is GLP compliant and there were methodological limitations in the study, including the low number of test animals and timing of blood sample collection and only one dose of glyphosate included. However, it should be noted that a significant delay in sexual maturation in male offspring (F1) indicated by delayed preputial separation (occurring after 45.9 days at 15000 ppm (top dose, higher than 1000 mg/kg bw/d) versus 43.0 days in the control group) was observed in the regulatory study CA 5.6.1/001-003 (2007). This was not considered to be related to changes in F1 male bodyweight. The delayed onset of sexual maturation did not have any impact on subsequent reproductive performance in the F1 generation. Thus, in a weight-of-evidence assessment the effects reported on male sexual development are not considered by RAC to be sufficient for a classification for effects on sexual function and fertility.

In the study by Panzacchi *et al.* (2018), Sprague-Dawley rats were exposed orally via drinking water to 1.75 mg glyphosate/kg bw/d starting from GD6. One cohort was dosed until sexual maturity (6-week cohort) and another cohort was dosed until adulthood (13-week cohort). No effects were reported on survival, body weights, food and water consumption following exposure to glyphosate. No clinical changes were reported. Furthermore, litter sizes were comparable among groups. It is noted that the study was not performed according to guideline or GLP compliant and there are methodological limitations of the study, including only one dose of glyphosate tested and only 8 females per dose group.

In the study by Ren *et al.* (2019), effects on lipid metabolism in foetuses and pups following prenatal exposure to glyphosate were investigated. Ten ICR mice/group were exposed via drinking water containing 0.5% glyphosate from GD1 to GD19 (corresponds to 1000 mg/kg bw/d using EFSA guidance document on default values). The control group received distilled water. Five dams/group were sacrificed on GD19, and foetuses were examined, while the remaining dams were allowed to litter and maintain their litters to PND21. Offspring (2/sex/litter where

possible) were selected on PND7 and 21 for evaluation. Foetal and offspring evaluations included liver histology, serum biochemistry, liver lipid concentration and gene expression analysis of genes related to lipid metabolism in the liver. Liver histopathology showed increased vacuoles with lipid droplets (more in female offspring compared to males), more red areas representing lipid substances and clusters of monocytes (PND7 females). The results of the study show some changes in lipid metabolism in the offspring exposed prenatally to glyphosate, however the clinical relevance of this finding is lacking, and no firm conclusion can be made due to the uncertainties such as low number of animals examined. It is noted that the study was not performed according to guideline or GLP compliant and there are methodological limitations of the study including that glyphosate was not sufficiently characterised, only one dose level was tested, there was large inter animal variability reported and few animals per group were analysed. RAC notes these limitations of the study.

During the consultation (comment no. 90) the study by Lorenz *et al.* (2020) was included. Lorenz *et al.* studied the effect of perinatal oral exposure to 2 mg glyphosate/kg bw/d from GD9 to weaning (PND21) on female fertility and the hormonal and uterine milieu in Wistar rats (F0: 11/group and F1: 21/group). F1 females (with no exposure to glyphosate from PND21) were followed to PND90 and then mated to untreated males. Results: F0 females: No effects on fertility and no effects in female pups were reported including an assessment of vaginal opening. Results F1 females: No effects on the pregnancy rate were reported following perinatal exposure to glyphosate. On GD19 no changes in the number of corpora lutea and in the number of resorptions were seen. However, in glyphosate exposed rats a statistically significant decreased number of implantation sites were reported compared to the control group (actual number of implantation sites per animals were not reported and the number of corpora lutea, resorption sites and preimplantation loss are only reported in graphics). Hormone levels were assessed at GD5 and glyphosate exposure induced higher 17β-oestradiol serum levels, without changes in progesterone. Further, glyphosate increased uterine ERα protein expression, with no differences at transcript level, and decreased the progesterone mRNA expression. Moreover, glyphosate downregulated Hoxa10 and Lif genes, with no difference in Muc1 and Areg expression, genes involved to sustain endometrial receptivity. RAC notes that only one low dose of glyphosate was tested, and that effects on female fertility were not reported in Wistar rats in OECD and GLP compliant reproductive toxicity studies at higher doses of glyphosate.

Overall summary

Effects on sperm parameters

In the study CA 5.6.1/001-003 (2007) a significant decrease in homogenisation resistant spermatids in cauda epididymis was reported in F0 males (309.0 million/gram compared to 399.9 million/gram in control) at the highest dose level of 15000 ppm (~1000 mg/kg bw/d). No significant effects were observed in the F1 generation. Sperm changes and histopathological examinations did not reveal any changes in the testis or epididymis. No general toxicity was observed in males. The decrease in homogenisation resistant spermatids in cauda epididymis was not confirmed in the study CA 5.6.1/005 (1997) where the same rat strain and dosages above 2000 mg/kg bw/d were used, or in the study CA 5.6.1/004 (2000) using another strain of rat and dose levels up to 10000 ppm (~1000 mg/kg bw/d). Dai *et al.* (2016) reported a decreased total sperm count in Sprague Dawley rats after 5 weeks exposure to 500 mg/kg bw glyphosate/d. However, this study was reliable with restrictions due to limited number of animals used and parameters investigated. A number of more recent *in vitro* and *in vivo* studies did not show clear evidence of effects on sperm and testis parameters in rats and mice.

Overall, after a review of the data and in a weight of evidence assessment effects on sperm parameters were observed, however, not consistently reported in the studies. The findings were confined to high doses (at or above the limit dose) and also in some studies in the presence of

general toxicity, or in studies with limited reporting. Thus, the available data do not provide some or clear evidence for adverse effects on sexual function and fertility.

Reduced litter size

The study CA 5.6.1/010 (1990) reported a slight reduction in the average litter size of 13% in the F0 dams at 30000 ppm (above 2000 mg/kg bw/d), and to a lesser degree in the F1 dams. The reduction was not statistically significant and not observed when F1 animals were re-mated. Maternal toxicity consisted of soft stool and reduced body weight. A reduction in litter size was not confirmed in the study CA 5.6.1/005 (1997) using the same rat strain of rat and the same dietary concentrations of glyphosate.

Overall, after a review of the data and in a weight of evidence assessment equivocal reduction in litter size was observed, however, at very high dose level (above 2000 mg/kg bw/d) and not confirmed in other studies. Thus, the data do not provide clear evidence for adverse effects on sexual function and fertility.

Delayed sexual maturation

In the study CA 5.6.1/001-003 (2007) delayed preputial separation was observed in F1 male offspring at 15000 ppm (~1000 mg/kg bw/d) (days at completion: 45.9 compared to 43.0 in control). No impact on subsequent reproductive performance was observed. General toxicity including liver and kidney weight changes were observed in parental females at 15000 ppm.

Manservisi *et al.* (2019) observed an increased anogenital distance in male Sprague-Dawley rats on PND4 following administration to glyphosate diluted in drinking water at 1.75 mg/kg bw/d. However, this study was reliable with restrictions due to low number of test animals and only one dose of glyphosate tested.

Overall, after a review of the data and in a weight of evidence assessment there are some data indicating effects on male sexual development, but the findings were confined to a dose level around the limit dose of 1000 mg/kg bw/d. Thus, data do not provide clear evidence for adverse effects on sexual function and fertility.

Lower fertility indices in females

In the study CA 5.6.1/005 (1997) lower fertility indices were observed in F1 females of high dose group (above 2000 mg/kg bw/d), however not statistically significant (79.2% compared to 95.8% in control). The finding was observed in the presence of general toxicity. The effects reported on oocytes in the three *in vitro* mechanistic studies are by RAC considered to be of limited *in vivo* relevance and are not considered to indicate some or clear evidence of adverse effects on female fertility.

Overall, RAC is of the opinion that in a weight of evidence assessment and the review of the available information including published literature which was not included in the previous evaluation by RAC in 2017 does not provide sufficient evidence to conclude that there is some or clear adverse effects of glyphosate on sexual function and fertility.

Comparison with the CLP criteria

Repr. 1A

There are no clear indications of effects on fertility or sexual function following exposure of glyphosate to humans, therefore RAC considers that a classification of glyphosate with Repr. 1A is not justified.

<u>Repr. 1B</u>

According to the CLP criteria, classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on reproductive toxicity in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects.

<u>Repr. 2</u>

According to the CLP criteria, classification of a substance in Category 2 is justified when there is some evidence from humans or experimental animals, possibly supplemented with other information of an adverse effect on sexual function and fertility, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be a more appropriate classification.

RAC concludes that the six two-generation reproductive toxicity studies and the study by Dai *et al.* (2016) did not provide any consistent evidence of effects of glyphosate exposure on fertility or on the male and female reproductive organs. Furthermore, no clear effects on sexual maturation in males and females was reported in the studies where this parameter was assessed. The effects seen were of equivocal relevance and were confined to high dose levels (> 1000 mg/kg bw/d) and were seen in the presence of parental toxicity. RAC further notes that the review of more recent studies from the open literature does not provide sufficient evidence to change the previous conclusion by RAC for adverse effects for sexual function and fertility due to limitations in the reporting of the studies, the lack of consistency in the results from the studies, and limited number of animals and doses of glyphosate used in the studies. Thus, a classification as Repr. 1B or Repr. 2 is not considered justified and **no classification for adverse effects on sexual function and fertility is warranted**.

Adverse effects on development

Studies in rats

The DS included five developmental toxicity studies in rats and seven studies in rabbits in their evaluation of developmental toxicity following exposure to glyphosate. In addition, a developmental neurotoxicity study with glyphosate trimesium was submitted by EFSA during the process (Moxon, 2001). It should be noted that RAC also assessed the original full study reports (robust study summaries are included in the RAR, B-6.6). The studies in rats are summarised in table below:

Table: *Developmental toxicity studies in rats. All studies were already included in the previous RAC opinion (CLH, 2016, RAC, 2017).*

Study, purity of glyphosate (study quality)	Strain, route, duration of treatment	Dose levels	NOAEL	LOAEL	Targets/ Main effects
CA5.6.2/001, 1996; 95.6% OECD TG 414 (1981) (Acceptable in RAR)	Alpk rats (Wistar derived) Gavage GD7-16	0, 250, 500, 1000 mg/kg bw/d	Maternal & developmental: 1000 mg/kg bw/d	Not applicable	None
CA 5.6.2/002, 1995; 95.68%	CD (SD) rats Gavage GD6-15	0, 30, 300, 1000	Maternal: 300 mg/kg bw/d	Maternal & developmental: 1000 mg/kg bw/d	Maternal: Loose stool Development: skeletal anomalies seen in all doses

Study, purity of glyphosate (study quality)	Strain, route, duration of treatment	Dose levels	NOAEL	LOAEL	Targets/ Main effects
OECD TG 414 (1981) (Acceptable in RAR)		mg/kg bw/d	Developmental: 1000 mg/kg bw/d		but not considered treatment related. Ventricular septal defects: One foetus each of the 300 and 1000 mg/kg bw/d. Right aortic arch: one foetus 300 mg/kg bw/d group (different foetus from the one with ventricular septal defects.
CA 5.6.2/003, 1991; 98.6% OECD TG 414 (1981) (Acceptable in RAR)	CD rats Gavage GD6-15	0, 300, 1000, 3500 mg/kg bw/d	Maternal & developmental: 300 mg/kg bw/d	Maternal & developmental: 1000 mg/kg bw/d	Maternal: high dose group: two deaths, bw gain (32%), noisy respiration (15/22) and gaseous distension in GI tract (2/25); Development: ossification (35.7% in high dose group, 28.4% in mid dose group, compared to 11.7% in control), skeletal anomalies at low incidences
CA 5.6.2/004-005, 1991; 96.8% Guideline not stated (Supplementary in RAR)	Wistar rats 30 x controls, 25 x treated group Gavage GD6-15 pre-GLP	0, 1000 mg/kg bw/d	Maternal: 1000 mg/kg bw/d; Developmental: < 1000 mg/kg bw/d	Maternal: not applicable; Developmental: 1000 mg/kg bw/d	Maternal: no effects; Development: ossification↓
CA 5.6.2/008, 1980; 98.7% OECD TG 414 (1981) (Acceptable in RAR)	Charles River rats Gavage GD6-19	0, 300, 1000, 3500 mg/kg bw/d	Maternal & developmental: 1000 mg/kg bw/d	Maternal & developmental. 3500 mg/kg bw/d	Maternal: mortality, soft stool, diarrhoea; Development: ↓ bw, ↑malformations

Four of the five studies reported no evidence of developmental toxicity in rats. Only two of the studies reported results that required an in-depth analysis of the data by RAC (CA 5.6.2/008, 1980; CA 5.6.2/003, 1991). RAC notes that for developmental toxicity in rats, no new information has been provided since the previous assessment by RAC in 2017.

The study CA 5.6.2/008 (1980) tested doses up to 3500 mg/kg bw/d. At this very high dose, excessive maternal toxicity was reported including mortality (6/25 dams died). Up to the limit dose of 1000 mg/kg bw/d only weak maternal effects such as gastrointestinal signs including soft stool and diarrhoea, or a lower bodyweight gain were seen. No increase in post-implantation losses were observed. The mean number was 0.6 ± 0.09, 0.2 ± 0.52, 0.5 ± 0.81 and 1.2 ± 1.25 in the 0, 300, 1000 and 3500 mg/kg bw/d dose groups, respectively. The foetal body weight was statistically significantly reduced at 3500 mg/kg bw/d (3.5, 3.7, 3.6 and 3.2 g at 0, 300, 1000 and 3500 mg/kg bw/d, respectively). The number of malformed foetuses were as follows (foetuses/litter): skeletal malformations: 1/1, 0/0, 0/0 and 9/2 and visceral malformations: 2/2, 0/0, 0/0, 7/2 at 0, 300, 1000 and 3500 mg/kg bw/d, respectively. In the high dose group, the malformations included six foetuses from one litter with a syndrome of bent tail, open eyelids, missing kidneys and ureters as well as various skeletal effects. Three foetuses in another litter were reported to have dwarfism. All the malformations were reported to be within the HCD range.

RAC concludes that the effects reported were seen at a very high dose levels (3500 mg/kg bw/d) that caused excessive maternal toxicity (~25% of the dams died during the study). According to the CLP criteria (Annex I, 3.7.2.4.4) data from a dose level with such an excessive toxicity should normally not be considered for further evaluation.

In the study CA 5.6.2/003 (1991) with exposure to glyphosate at 0, 300, 1000, 3500 mg/kg bw/d, maternal toxicity was evident at the high dose level as two mortalities and signs of salivation post-dosing, wet coats, noisy respiration/gasping and loose faeces as well as gaseous distention of the GI tract. A marked reduction in body weight gain during the first two days of treatment and a slight reduction in body weight gain during GD12-14 was also reported together with a reduced food intake during the dosing period. In the mid dose group, noisy respiration was reported in 2/25 dams together with a slight reduction in bw gain during the 2 first days of dosing. A total of 23, 23, 25 and 22 dams had live pups at GD20 in the control, 300, 1000 and 3500 mg/kg bw/d dose groups, respectively. There were no abortions and no total resorptions. Implantation rate, post-implantation loss and litter size were similar in all groups. Evidence of delayed ossification, increased incidence of foetuses with wavy ribs and reduced foetal weight was recorded at 1000 mg/kg bw/d (table below). RAC considers that the effects on foetal weight and on the degree of ossification are secondary effects, due to the maternal toxicity observed in the high dose group and notes that an increase in wavy ribs was not recorded in any of the other available developmental toxicity studies in rats. A total of 1 foetus from 1 litter, 2 from 2 litters, 1 from 1 litter, and 3 from 2 litters in the control, 300, 1000 and 3500 mg/kg bw/d dose groups, respectively, were malformed (foetal incidence: 0.3, 0.8, 0.3 and 1.1%, respectively). The malformations observed were as follows: in the control group there was one foetus with markedly distended urinary bladder. In the 300 mg/kg bw/d group there was one small foetus (2.24 g vs approximately 4 g in control group) with left microphthalmia and one foetus with termination of vertebral column at the 1st sacral vertebra. These two foetuses were from different litters. In the 1000 mg/kg bw/d group one foetus had an interventricular septal defect and absent innominate artery. In the 3500 mg/kg bw/d group there was one small foetus (1.53 g) with an interventricular septal defect, palatine irregularity, nasopharyngeal fistula and subcutaneous oedema and atelectatic lungs; one foetus with palatine irregularity with misshapen basisphenoid and connected 5th to 6th right cervical vertebral arches; and one foetus with cervical irregularities, including one absent right, shortened 1st left and reduced ossification of cervical vertebral arches. RAC notes that a minimal increase in the foetal incidence of malformations was reported in the high dose group (see above). However, these were not statistically significant and showed no dose-response relationship for the single incidences of ventricular septal defect in the mid and high dose groups. RAC therefore concludes that no evidence of developmental toxicity was reported in this study.

Table: *Foetal effects attributable to treatment in rats (CA 5.6.2/003, 1991)*

Parameter	Dose level (mg/kg bw/d)			
	0	**300**	**1000**	**3500**
Mean foetal weigh (g)	3.96	3.90	3.89	3.71**
Foetuses with wavy ribs (thoracic ribs) / number of foetuses examined	1/155	-/143	3/166	28/144
Reduced ossification of 1 or more cranial centres	3/155	2/143	12/166	10/144
Reduced ossification of sacrocaudal vertebral arches	3/155	8/143	17/166	15/144
Foetuses with unossified sternebrae (%)	13.7	28.5	17.6	33.8**
Foetuses showing skeletal variation (%)[1]	11.7	22.6	28.4	35.7**

* Statistically significant, $p < 0.05$; ** $p < 0.01$

[1] Historical control range for skeletal variations: 21.9 - 27.2%

CA 5.6.2/002 (1995) study with exposure to 0, 30, 300 or 1000 mg glyphosate/kg bw/d showed a slight increase in skeletal variations including lumbar ribs (11 foetuses from 7 litters compared to 4 foetuses from 2 litters in control animals) in the high dose group. External malformations included a short tail in one foetus of the 30 mg/kg bw/d group and microphthalmia in one foetus of the 1000 mg/kg bw/d group. Visceral examination revealed ventricular septal defects in one foetus of each of the 300 and 1000 mg/kg bw/d groups and another foetus (from a different litter) at 300 mg/kg bw/d displayed a right aortic arch. Skeletal malformations were rare and were not associated with treatment, the incidences being similar in all groups (2, 0, 2 and 3 foetuses had malformations in the control group, 30, 300 and 1000 mg/kg bw/d groups, respectively). The malformations included splitting of ossification centres of the thoracic vertebral bodies and asymmetry of the sternebrae with sternocostal joint displacement. During the dosing period in the 1000 mg/kg bw/d group, 20 out of 22 pregnant females showed slightly loose stool and the increase in its incidence was statistically significant. There were no mortalities. Maternal toxicity was considered as minimal. RAC concludes that no evidence of developmental toxicity was reported in this study.

CA 5.6.2/004-5 (1991) was a supplementary limit test in Wistar rats with only two groups; a control group and a 1000 mg/kg bw/d group. Mortality and clinical signs of toxicity were not evident. The incidence of foetal malformations was not increased relative to controls. A significantly increased incidence of delayed ossification (normal variations) including caudal vertebral arch, forelimb proximal phalange and hindlimb distal phalanges were reported at 1000 mg/kg bw/d. RAC concludes that this limit test did not result in any increased incidences of external, visceral or skeletal malformations.

The most recent study CA 5.6.2/001 (1996) showed no effects at doses up to 1000 mg/kg bw/d. One control animal was killed on day 7 due to mis-dosing. There was no evidence of maternal toxicity or effects on the foetuses. The incidence of foetuses with major defects was 1/284, 1/297, 1/301 and 2/296 in the control and 250, 500 and 1000 mg /kg bw/d groups, respectively. Neither the type nor incidence of major defects provided evidence for an adverse effect of glyphosate. The defects were dissimilar in type and of single incidence. Further, the proportion of foetuses with external/visceral variants and the proportion of foetuses with skeletal variants were lower in the glyphosate treated groups than in the control group. RAC concludes that no evidence of developmental toxicity attributable to glyphosate was reported in this study.

In the developmental neurotoxicity study, 30 Wistar rats/group were exposed to 0, 10, 25 or 100 mg/kg bw/d glyphosate trimesium (purity 57.4%) from GD7 to PND11. Four/sex/litter and 20/litter/dose were assessed (Moxon, 2001). The study was performed in compliance with GLP, however, with some deviations from the OECD TG 426. In the study, the following parameters were assessed: motor activity (PND14, 18, 22, 60), auditory startle response habituation (PND23 and 61), learning and memory (PND21 and 59), sexual maturation (males at PND41, females at PND29). The neural tissue was collected on PND12 and at study termination PND63 (control and high dose animals assessed). No maternal toxicity was reported (LOAEL > 100 mg/kg bw/d). Foetal toxicity included: decreased motor activity at PND14: males; 72% and 70% compared to controls in mid and high dose. Females; 65% and 45% compared to controls in mid and high dose. Effects on learning and memory were assessed in the water maze test, where the high dose males responded statistically significantly slower to finding the escape ladder compared to controls. The pup survival was decreased by 19% on PND5 in high dose group compared to controls. During PND1-5 the pup survival was 84.8% in high dose group compared to controls. Pup body weight gain was decreased by 10% during PND1-5, however, with no changes between groups post- weaning. No effects on sexual maturation and brain weight were reported. Further, there were no neuropathological findings recorded in the study. RAC notes the very low purity of glyphosate trimesium (57.4%) used in the study, making it difficult to assess if the effects reported were related to glyphosate exposure, or to the impurities in glyphosate trimesium.

Further, glyphosate trimesium was not one of the glyphosate salts included in the CLH dossier by the DS, however, as no developmental neurotoxicity study was available for glyphosate it was decided to include it in the opinion. In addition, it was noted that the Applicant informed that glyphosate trimesium has not been manufactured since 2003 and not sold since 2004 and that was regulated as a separate active ingredient to glyphosate acid itself. RAC considers that due to the limitations in the study, it has no major impact on the classification of glyphosate for developmental toxicity.

Summary of rat developmental toxicity studies

In one of the five studies in rats (CA 5.6.2/008, 1980) malformations were observed (reported within the HCD range) at a very high dose level (3500 mg/kg bw/d) that caused excessive maternal toxicity (~25% of the dams died during the study). According to the CLP Regulation (Annex I, 3.7.2.4.4) data from a dose level with such an excessive toxicity should normally not be considered for further evaluation. RAC concludes that no classification for development is justified according to the CLP criteria based on this study.

Cardiovascular malformations were reported in two of the five studies with rats. In the study CA 5.6.2/002 (1995), they were reported as single incidences at 300 and 1000 mg/kg bw/d and were not considered related to maternal toxicity. In the study CA 5.6.2/003 (1991), a single incidences of cardiovascular malformations was reported at 1000 and 3500 mg/kg bw/d in the presence of maternal toxicity only at 3500 mg/kg bw/d. RAC concludes that due to the single incidences of cardiovascular malformations without a dose-response relationship and without statistical significance in the six rat developmental toxicity studies, no classification for development is justified according to the CLP criteria based on the studies in rats.

RAC notes that no effects relevant for classification for developmental toxicity were reported in the 2-generation reproductive toxicity studies with glyphosate. The effects reported included decreased pup bodyweight and caecum distention at doses > 2000 mg/kg bw/d.

Studies in rabbits

In the table below, the main effects seen in the eight developmental toxicity studies in rabbits following exposure to glyphosate are summarised. Further information on maternal toxicity is included in the STOT RE section in the table: "Rabbit maternal mortality and toxicity from developmental studies with glyphosate".

Table: *Developmental toxicity studies in rabbits[1] All studies were already included in the previous RAC opinion (CLH, 2016, RAC, 2017), unless specified otherwise.*

Study, purity of glyphosate (study quality)	Strain, route, duration of treatment	Dose levels	NOAEL	LOAEL	Targets/ Main effects
CA 5.6.2/010, 1996; 95.3% GLP (study acceptable in RAR)	NZW rabbit 18 rabbits/dose group Gavage GD7-19	0, 50, 200, 400 mg/kg bw/d	Maternal & developmental: 50 mg/kg bw/d	Maternal & developmental: 200 mg/kg bw/d	Maternal effects at the high dose: diarrhoea and scours, mortality (2 deaths), stat. sign. ↓ bw gain and food consumption; Development: stat. sign. ↑ post-implantation loss at mid dose.
CA 5.6.2/009 1996; 95.6%	NZW rabbit	0, 100, 175, 300	Maternal: 100 mg/kg bw/d;	Maternal: 175 mg/kg bw/d;	Maternal: in high dose group, food intake ↓and stat.

Study, purity of glyphosate (study quality)	Strain, route, duration of treatment	Dose levels	NOAEL	LOAEL	Targets/ Main effects
GLP (study acceptable in RAR)	20 rabbits/dose group Gavage DG8-20	mg/kg bw/d	Developmental: 175 mg/kg bw/d	Developmental: 300 mg/kg bw/d	sign. bw gain ↓, diarrhoea; Development: foetal weight stat. sign. ↓ in high dose group, ossification retarded. Minor skeletal defects.
CA 5.6.2/011 1995; 97.6% GLP (study acceptable in RAR)	Japanese White rabbits (Kbl:JW) 18 rabbits/dose group Gavage GD6-18	0, 10, 100, 300 mg/kg bw/d	Maternal: 100 mg/kg bw/d; Developmental: 300 mg/kg bw/d	Maternal: 300 mg/kg bw/d; Developmental: not applicable	Maternal: mortality (1 death), loose stool, abortions (2 in low and high dose group). No effects on food intake or bw; Development: stat. sign. ↑ in % of litters with skeletal malformations at 300 mg/kg bw/d.
CA 5.6.2/012-013, 1993*; 96.8% GLP (study supplementary in RAR)	NZW rabbit 26, 17, 16 or 15 rabbits in the 0, 20, 100, 500 mg/kg bw/d dose groups Gavage GD6-18	0, 20, 100, 500 mg/kg bw/d	Maternal: 20 mg/kg bw/d; Developmental: 100 mg/kg bw/d	Maternal: 100 mg/kg bw/d; Developmental: not established due to low number of foetuses at top dose	Maternal: mortality (4 deaths at mid and 8 at high dose), soft/liquid stool; stat. sign. ↓ food consumption and bw and bw gain in high dose. Development: no clear-cut effects up to 100 mg/kg bw/d (in high dose group low number of foetuses and litters, but stat. sign. increase in visceral malformations in all dose groups (dilated heart).
CA 5.6.2/014, 1991; 98.6% GLP (study acceptable in RAR)	NZW rabbit 19, 19, 16 or 20 rabbits in the 0, 50, 150, 450 mg/kg bw/d dose groups Gavage GD7-19	0, 50, 150, 450 mg/kg bw/d	Maternal: 50 mg/kg bw/d; Developmental:150 mg/kg bw/d	Maternal: 150 mg/kg bw/d; Developmental: 450 mg/kg bw/d	Maternal: mortality following abortion (1 at top dose), clinical signs (GI-tract), food intake and bw gain ↓ Development: late embryonic death, post-implantation loss, cardiac malformations.
CA 5.6.2/016, 1989**; 95% Lot 38 Study has serious deficiencies. Not GLP (study not acceptable in RAR)	NZW rabbit 15 rabbits/dose group Gavage GD6-18	0, 125, 250, 500 mg/kg bw/d	Maternal & developmental: 250 mg/kg bw/d	Maternal & developmental: 500 mg/kg bw/d	Maternal effects in high dose: food intake stat. sign. ↓ and bw ↓, 2 abortions; Development: malformations (external, visceral & skeletal).

Study, purity of glyphosate (study quality)	Strain, route, duration of treatment	Dose levels	NOAEL	LOAEL	Targets/ Main effects
CA 5.6.2/018, 1980; 100% Pre guideline/GLP (Study supplementary in RAR) Not included in previous RAC opinion	Dutch belted rabbit 5/dose Gavage GD6-27	0, 125, 250, 500, 1250 and 2500 mg/kg bw/d	Maternal: Not applicable	Maternal/developmental: not applicable	Mortality: 4/5 at 500 mg/kg bw/d 5/5 at 1250 mg/kg bw/d 5/5 at 2500 mg/kg bw/d No maternal toxicity and no treatment related effects on pregnancy or development observed at 125 and 250 mg/kg bw/d. Doses of 500 mg/kg bw/d and higher clearly exceeded the MTD.
CA 5.6.2/019, 1980**; 98.7% Adhere to GLP (study supportive in RAR)	Dutch Belted rabbit 16/dose Gavage GD6-27	0, 75, 175, 350 mg/kg bw/d	Maternal: 75 mg/kg bw/d; Developmental: 175 mg/kg bw/d	Maternal: 175 mg/kg bw/d; Developmental: not established due to low number of foetuses	Maternal: mortality (1, 2 and 10 at low, mid and high dose), soft stool, diarrhoea. No effects on maternal bw and bw gain; Development: none up to 175 mg/kg bw/d (high dose group excluded and not assessed. Due to maternal mortality only 6 litters were available at c-section.

* Supplementary study since high dose group could not be evaluated for developmental toxicity/teratogenicity.

** Study with serious deficiencies in conduct and reporting.

[1] Detailed study summaries are included in the Volume 3 - B6.67 of the "Renewal assessment Report" (p 176 - 220).

The developmental toxicity studies showed that pregnant rabbits are more sensitive than pregnant rats to the exposure to glyphosate.

Severe maternal toxicity seen as treatment-related premature deaths was reported in several studies at doses ranging from 100 to 500 mg/kg bw/d. Many of the female rabbits that died or were killed in extremis seem to have severe effects in the GI tract including ulceration. A possible explanation for the greater sensitivity of pregnant rabbits compared to pregnant rats following exposure to glyphosate may be because rabbits ingest their caecotrophes (a specialised digestive strategy for the recycling of caecal contents and the extraction of nutrients). This may lead to two outcomes in the rabbits:

1. glyphosate as well as other substances that predominantly are excreted unchanged in the faeces, can be readily available for repeated oral uptake and the caecotrophs may therefore constitute a potential source of increased exposure to glyphosate in rabbits relative to other species, including humans. This possible recycling of glyphosate and increased exposure in rabbits might explain the particular sensitivity of this species;
2. maternal toxicity was reported as soft stools and diarrhoea and these effects may prevent the rabbits from ingesting their caecotrophs, and consequently the overall well-being of

the rabbits would be affected. Further information regarding the premature deaths is included in the table "Rabbit maternal mortality and toxicity from developmental studies with glyphosate" in the STOT RE section.

According to the CLP Regulation, maternal mortality greater than 10% is considered excessive and the data from this dose level shall not normally be considered further for evaluation (CLP Regulation, Annex I, 3.7.2.4.4). However, following exposure to glyphosate some of the premature deaths were reported to be related to treatment with glyphosate while others were due to mis-gavage or infections.

In the section below, the two studies requiring in-depth analysis for effects on foetal viability are summarised followed by the six studies requiring in depth analysis for foetal pathological findings.

Effects on foetal viability

Effects on embryo-foetal viability, which can be revealed by analysing a number of parameters (e.g., viable litter size at C-section, post-implantation loss, number of early and late embryo-foetal death and number of dead foetuses) that are interlinked in one way or another to each other, were only reported in two of the available studies, i.e., in CA 5.6.2/010 (1986) and in the CA 5.6.2/014 (1991) (see table A in the section "Supplemental information - in depth analysis by RAC" for an overview of the observed effects on foetal viability in the available rabbit developmental toxicity studies).

In the study CA 5.6.2/010 (1996), described as acceptable in the RAR and performed with NZW rabbits, a slightly increased number of post-implantation loss was recorded at the two highest dose levels. However, the dose-response relationship in the increase in post-implantation losses was not considered to be convincingly (mean % of post-implantation loss: 3.7 ± 6.5, 3.6 ± 8.5, 11.5 ± 11.4 and 12.1 ± 18.6 in the 0, 50, 200 and 400 mg/kg bw/d dose groups, respectively). In the high dose group (400 mg/kg bw/d) the slight, but not statistically significant, increase in late embryo/foetal deaths and post-implantation loss was considered not to be related to treatment, since it was mainly due to one animal that had nine late embryonic/foetal deaths (resulting in a post-implantation loss of 69.2% in that specific animal). In addition, the mean viable litter size at C-section was similar at all dose levels (9.1 ± 2.5, 8.7 ± 2.4, 7.9 ± 2.5 and 8.9 ± 2.6 in the control, low, intermediate and high dose group, respectively) and this effect observed at the intermediate dose level is considered to have limited biological relevance. Further, no dose-related or statistically significant effect was recorded on foetal weights at any dose levels up to and including 400 mg/kg bw/d (41.5 ± 5.5, 39.4 ± 5.6, 41.7 ± 4.5 and 38.2 ± 5.2 g in the control, low, intermediate and high dose groups, respectively). At the highest dose level, maternal toxicity was observed as a statistically significant decrease in body weight gain from GD10-29 with clinical signs that included diarrhoea and scours, as well as premature death of two female rabbits (one died at GD19, and one was killed in extremis on GD20). The macroscopic necropsy findings of the 2 female rabbits included fluid filled large intestines, haemorrhage, ulceration and sloughing of the stomach, congested duodenum and gas distended colon, rectum and appendix. In the intermediate dose (200 mg/kg bw/d), maternal toxicity was evident as a decrease in bw gain, however, it was not statistically significant. At this dose level one female was found dead on GD16 and necropsy findings in the lungs indicated that the death was due to technical complications during dosing. At the low dose, no mortality occurred. In the control group, one doe was found dead two minutes after dosing and necropsy findings in the lungs indicated mal-dosing. Overall, RAC concludes that the increase in post-implantation loss was of low biological relevance.

In the study CA 5.6.2/014 (1991), considered acceptable in the RAR, a similar degree of increase in post-implantation loss was recorded at all dose levels (19.5 ± 19.8, 15.3 ± 17.2 and 21 ± 11.8 at 50, 150 and 450 mg/kg bw/d, respectively), compared to controls (5.7 ± 7.2), see table

below. Although a dose-related decrease of the mean litter size at C-section was noted, the reduction in the litter size was small and not statistically significant. RAC notes the absence of a dose-response relationship for the post-implantation loss and that according to the available HCD (based on 21 studies performed during 1989 and 1990; range: 6.5 - 17.5; median 12.9) there was a great variability in post-implantation loss in rabbits in the test facility where this study was performed. Maternal toxicity was reported as one maternal death at the top dose of 450 mg/kg bw/d on GD20 following abortion, gastrointestinal disturbances, reduced food intake and pronounced body weight loss (-660 g) as well as few haemorrhagic depressions in the stomach. Female rabbits that survived in the two highest dose groups showed reduced food consumption compared to the controls, but these were not statistically significant. In the mid dose at 150 mg/kg bw/d, a reduction in body weight of 12% compared to controls was observed from GD11-19. At 450 mg/kg bw/d this was also evident throughout the treatment period with reductions of 6 - 17% during GD7-19. No statistically significant effect on absolute maternal bw was recorded throughout the study, but a slight decrease in bw gain that coincided with the reduction in food consumption was recorded during GD11-20 at the mid dose (-32% less than controls) and top dose (-46%), respectively. However, RAC notes that according to CLP Regulation (Annex I, 3.7.2.4): "*In rabbits, the body weight gain may not be useful indicators of maternal toxicity because of normal fluctuations in body weight during pregnancy*". A dose related increase in females showing soft/liquid faeces were seen at the two highest doses.

Table: *Summary of maternal and litter parameters (group mean values) in rabbits from the study CA 5.6.2/014 (1991).*

Parameter	Dose group (mg/kg bw/d)				Historical control range (mean value)
	0 (control)	**50**	**150**	**450**	
No. of mated females	19	19	16	20	--
No. not pregnant	0	6	1	5	--
No. of premature deaths	0	0	0	1§	
No. of female rabbits with live pups or litters at day 29	18	12	15	13	--
Reduced faecal output	9	8	11	12	
Soft/liquid faeces	0	2	5	13	--
Corpora lutea	11.5	12.4	11.7	11.3	9.0 - 12.9 (11.2)
implantations	9.7	10.5	9.0	9.2	7.0 - 11.1 (9.5)
Pre-implantation loss	14.6	15.4	23.4	18.8	2.3 - 26.1 (15.1)
Early embryonic deaths	0.4	0.9	0.9	0.5	0.3 - 1.1 (0.6)
Late embryonic deaths	**0.2**	**0.9**	**0.5**	**1.3****	**0.1 - 1.3 (0.7)**
Abortions	0.0	0.0	0.1	0.0$^\#$	0.0 - 0.1 (0)
Total embryonic deaths	**0.6**	**1.8***	**1.5***	**1.8****	**0.6 - 2.0 (1.2)**
Post-implantation loss (%)	**5.7**	**19.5***	**15.3***	**21.0****	**6.5 - 17.5 (12.9)*****
Live pups	9.1	8.7	7.5	7.3	6.1 - 9.5 (8.3)
Litter weight (g)	389.5	370.6	320.5	315.0	281.9 - 402.2 (352.9)
Mean foetal weight (g)	43.9	43.3	44.0	44.5	41.4 - 47.6 (44.1)
Sex (% males)	55.3	55.8	57.6	53.8	--

§ Day 20, following abortion on the day before.

* Statistically significant by Kruskal-Wallis 'H' test p < 0.05.

** Statistically significant by Kruskal-Wallis 'H' test p < 0.01.

*** HCD: 8.1% (2.8 - 17.7) Holson *et al.*, 2006 and 9.1% (0.6 - 23.4) (MARTA, 1997).

Fisher's exact test follow up by intergroup comparison with control was not statistically significant p > 0.05.

No similar effect on post-implantation loss were recorded in the studies CA 5.6.2/009 (1996) and CA 5.6.2/011 (1995) where dose levels up to 300 mg/kg bw/d was used, or in the study CA 5.6.2/012-013 (1993) with dose levels up to 500 mg/kg bw/d. In the study CA 5.6.2/016 (1989) where dose levels up to 500 mg/kg bw/d were used, a slightly higher mean number of embryo/foetal death (1.4 ± 2.20 as compared to 0.07 ± 0.26 in the control) and a slightly lower mean number of viable implants/litter (5.2 ± 3.03 as compared to 7.3 ± 3.1 in the control) was reported. However, the study CA 5.6.2/016 (1989) (not acceptable in RAR B-6.6) had serious deficiencies in conducting and reporting, no statistical analysis was provided and since data from the 2 high dose dams that aborted during the study were included in the analysis; it is not clear to what extent this data influenced the outcome of the data analysis. Consequently, the data from this study should be handled with caution and will not be taken into account in the overall weight of evidence analysis.

Overall, RAC concludes that following *in utero* exposure to glyphosate in rabbits no clear relationship between exposure and effects on foetal viability could be determined. Effects on foetal viability were not reported consistently in the four acceptable developmental toxicity studies in rabbits. Actually, only one study (CA 5.6.2/014, 1991) reported effects on foetal viability, however, without a dose-response and within the historical control range for late and total embryonic deaths.

Foetal pathological findings

An overview of the observed foetal pathological effects is presented in table B in the section "Supplementary information - in depth analysis by RAC".

In five out of eight developmental toxicity studies performed in rabbits, foetal skeletal and visceral malformations were reported, but at low incidences and, in the study where HCD were available (CA 5.6.2/014, 1991), they were within the range of the HCD. The foetal skeletal and visceral malformations were also reported in the presence of severe maternal toxicity including death and GI tract intolerance. However, the deaths were reported to be both substance-related and due to technical problems with the dosing of the animals or related to infections. An assessment of the five studies is included below.

In the study CA 5.6.2/009 (1996), acceptable in the RAR B-6.6 and performed with NZW rabbits, the number of foetuses (litters) with skeletal malformations were 3(2), 0, 0 and 1(1) in the controls, 100, 175 and 300 mg/kg bw/d dose groups, respectively, and the number of foetuses (litter) with external and visceral malformations were 2(2), 1(1), 0 and 2(2) in the controls, 100, 175 and 300 mg/kg bw/d dose groups, respectively. One foetus at the 100 and 300 mg/kg bw/d dose levels was reported to have a single heart ventricle, thickened ventricle walls, enlarged aorta and reduced pulmonary artery, whereas one control foetus was reported to have an enlarged aorta and a persistent truncus arteriosus. In the high dose group, there was also one foetus with gross malformations of the skull. A statistically significant increase in foetuses (litter) with minor skeletal defects was reported in the low and high dose group (58(16), 82(18), 59(16) and 79(17) at 0, 100, 175 and 300 mg/kg bw/d). However, when looking at the individual minor skeletal effects, a statistically significant increase was recorded only in the high dose group for the following observations: partially ossified transverse process on the 7th cervical vertebrae (8 foetuses in 2 litters as compared to 1 foetus in the controls), unossified transverse process on the 7th lumbar vertebrae (14 foetuses in 4 litters as compared to 4 foetuses in 3 litters in the controls) or partially ossified 6th sternebrae (16 foetuses from 7 litters as compared to 4 foetuses in 2 litters in the controls). It should also be noted that the foetal bw was statistically significantly reduced in the top dose group (44.4 g in controls and 40.7 g at 300 mg/kg bw/d). A statistically significant increase in foetuses (litter) with skeletal variations was also reported in the high dose group (119(17), 129(18), 116(17) and 132(17) at 0, 100, 175 and 300 mg/kg bw/d). These

variations included an increase (but not statistically significant) in the incidence of foetuses with partially ossified odontoids (62 foetuses in 15 litters as compared to 50 foetuses in 15 litters in the controls) or 27 pre-sacral vertebrae (37 foetuses in 12 litters as compared to 23 foetuses in 10 litters in the controls). Abortions occurred in 1, 2, 1 and 2 rabbits in the 0, 100, 175 and 300 mg/kg bw/d dose groups, respectively. All animals that aborted died or were sacrificed in extremis. In the high dose group, a statistically significant reduction in maternal body weight gain was reported and was accompanied by a reduction in food consumption. However, RAC notes that as described above the "*in rabbits, the body weight gain may not be useful indicators of maternal toxicity because of normal fluctuations in body weight during pregnancy*". RAC concludes that the minor and major defects did not show a clear dose-response with increasing dose, and were also reported in the control group, and therefore not considered related to treatment.

As revealed by table B (see Supplementary information section, and in table B6.6-52 in Annex 7 to the RAR), the main finding at the external visceral and skeletal examination in the study CA 5.6.2/012-013 (1993) considered to be supplementary in the RAR B-6.6 was cardiovascular malformations (summarised in the table below). This study using NZW rabbits, showed that the percentage of foetuses with "dilated heart" was significantly increased at all dose levels. At 20 mg/kg bw/d, 4 cases of dilated heart were reported with 2 cases in one litter and 1 case in each of 2 litters. At 100 mg/kg bw/d, 3 cases of dilated heart were reported in 1 litter and 1 case in another litter, and at 500 mg/kg bw/d 4 cases of dilated heart was reported in one litter and 1 case in another litter. No definition of the recorded dilated heart or information regarding the HCD for dilated heart was included by the DS or in the study report. Foetal weights were statistically significantly increased in the low and mid dose groups (32, 35, 35, 33 g in the 0, 20, 100, 500 mg/kg bw/d dose groups, respectively). There were no significant maternal effects in the dam with 3 cases of dilated heart at 100 mg/kg bw/d. In the dam with 4 cases of dilated heart at 500 mg/kg bw/d, soft stool and diarrhoea was recorded at GD10. Further information regarding maternal toxicity included that 4/16 females in the mid dose and 5/15 females in the high dose group died during the dosing period (table below). In addition, 3 females in the high dose died after cessation of substance administration. It is noted that in the control group two females also died, however, this was considered to be due to mis-dosing during gavage. Some uncertainties are also described relating to the cause of the premature death in the 100 and 500 mg/kg bw/d dose groups since various findings in the lungs and trachea, suggestive of gavage errors, were recorded at gross necropsy in 5/8 (high dose) and in 1/4 (intermediate dose) female rabbits. These findings may indicate that the premature death may be related to gavage errors but the unclear findings following necropsy in some of these animals makes this inconclusive. RAC concludes that the high incidence of maternal deaths is considered to lead to an insufficient number of foetuses being available for assessment from the high dose group (i.e., 28 foetuses from 5 litters). Further, RAC considers that the reporting of cardiovascular malformations was insufficient due to a lack of measurements of the heart and that no definition of the diagnosis was provided in the study report. No information regarding the HCD for dilated heart was included by the DS or provided in the study report.

Table: *Summary of mortality in female rabbits in the study CA 5.6.2/012-013 (1993)*

Parameter	Dose group (mg/kg bw/d)			
	0 (control)	**20**	**100**	**500**
Mated females	26	17	16	15
Dead during treatment	1*	0	4	5
Died post-treatment	1*	0	0	3
Total number of deaths	2	0	4***	8**
% mortality	7.7	0.0	25.0	53.3

* Animal died due to mis-gavage

** 5 out of 8 female rabbits had lung lesions (emphysema, collapsed, pneumonic lesions, consolidated and congested)

***1 out of 4 female rabbits that died had lung and trachea congestion and froth in trachea

Table: *Cardiovascular malformations in the rabbit study CA 5.6.2/012-013 (1993).*

Parameter	Dose groups (mg/kg bw/d)			
	0 (control)	20	100	500
No. of foetuses/no. of litters examined	133/20	78/13	77/12	28/5
Major visceral malformations:				
No. of foetuses/litters with dilated heart	-	4*/3	4*/2	5*/2*
No. of foetuses/litters with cardiomegaly	0	0	1[A]	0
No. of foetuses/litters with "seal shaped" hearts	1/1	0	1[A]	0
No. of foetuses/litters with dilated ventricle	1/1	0	1/1	1/1
No. affected/total no. of foetuses	2/133	4/78	7/77	5/28
Litters affected/total no. of litters	2/133	3/13	2/12	2/5

* Statistically significant, p ≥ 0.05

A Same foetus

In the study CA 5.6.2/014 (1991) (described as acceptable in the RAR B-6.6) performed with NZW rabbits, the number of foetuses, % (litters, %) with major malformations were 3, 1.9% (3, 16.67%), 3, 5.8% (3, 25%), 5, 4.3% (3, 20%) and 6, 5.9% (5, 38.5%) in the control, 50, 150 and 450 mg/kg bw/d dose groups. Single incidences (usually only found at one dose level) of some major malformations were identified in the cranial, lumbar or lumbar/sacral region of the foetus. Mal-rotated hindlimbs/forelimb flexure and/or hindlimb/forelimb brachydactyly were also reported with a foetal (litter) incidence of: 0, 2(2), 1(1) and 1(1) at the control, low, mid and high dose levels, respectively. However, the main finding in the study CA 5.6.2/014 (1991) was the recording of different cardiovascular malformations (see table below). Interventricular septal defects were recorded at the highest dose and were seen in 4 foetuses from 4 litters (i.e., at an incidence outside the HCD). The same effects were seen in one foetus from each of the other dose groups, including the control group. Other cardiovascular malformations of low incidence (but still outside the HCD) were: enlarged left ventricles, reduced right ventricles, retro-oesophageal right subclavian artery and narrow/dilated aortic arch/pulmonary trunk/arterial trunk. It should, however, be noted that in the high dose group interventricular septal defect, enlarged left, reduced right ventricles and narrow/dilated aortic arch/pulmonary trunk/arterial trunk originated from two foetuses from two different litters. Retro-oesophageal right subclavian artery was reported in two foetuses from the same litter, one of these foetuses were also reported to have interventricular septal defect. Thus, the cardiovascular malformations were to some extent clustered together in the same foetuses. In the mid dose group, all three foetuses with retro-oesophageal right subclavian artery were from the same litter (see table below). Maternal toxicity was reported as one maternal death at the top dose of 450 mg/kg bw/d on GD20 following abortion, GI disturbances, reduced food intake and body weight loss. Females in the two highest dose groups showed reduced food consumption compared to the controls, but these were not statistically significant. In the mid dose at 150 mg/kg bw/d a reduction of 12% was observed from GD11-19. At 450 mg/kg bw/d this was also evident throughout the treatment period with reductions of 6 - 17% during GD7-19. No changes in maternal bw throughout gestation were reported. A dose related increase in females showing soft/liquid faeces and signs of lack of appetite were seen at the two highest doses. However, in the top dose group there was no clear correlation between the severity of the maternal toxicity and the foetuses with interventricular

septal defects. RAC concludes that the reported increase in cardiovascular malformations were to some extent clustered together in the same foetuses and was shown in the presence of maternal toxicity, however, it was not considered marked.

Table: Summary of foetal parameters in rabbits in the study CA 5.6.2/014 (1991).

Parameter	Dose group (mg/kg bw/d)				Historical control range or x/y ◊ (mean)
	0 (control)	50	150	450	
Number of female rabbits with live pups or litters at day 29	18	12	15	13	--
Mean foetal weight (g)	43.9	43.3	44.0	44.5	41.4 - 47.6 (44.1)
Sex (% males)	55.3	55.8	57.6	53.8	--
Malformations					--
Total number of foetuses examined	163	104	112	95	1511
Number of malformed foetuses (%)	3 (1.9)	3 (5.8)	5 (4.3)	6 (5.9 (F))	51 (0.7 - 5.9 (3.8))
Number of affected litters (%)	3 (16.67)	3 (25)	3 (20)	5 (38.5)	43/188 (22.9)
Cardiovascular malformations					--
Number of foetuses with interventricular septal defect (%)	1K (0.6)	1J (1.0)	1F (0.9)	4A,B,C,D (4.2)	10/1511 (0.66)
Litter incidence (%)	1 (5.6)	1 (8.3)	1 (6.7)	4 (30.8)	10/188 (5.3)
Foetuses with enlarged left, reduced right ventricles (%)	0 (0)	0 (0)	0 (0)	2B,D (2.1)	2/1511 (0.13)
Litter incidence (%)	0 (0)	0 (0)	0 (0)	2 (15.4)	2/188 (1.10)
Foetuses with retro-oesophageal right subclavian artery (%)*	0 (0)	0 (0)	3G,H,I (2.7)	2A,E (2.1)	7/1511 (0.46)
Litter incidence (%)	0 (0)	0 (0)	1 (6.6)	1 (7.6)	7/188 (3.72)
Foetuses with narrow/dilated aortic arch/pulmonary trunk/arterial trunk (%)	1K (0.6)	1J (1.0)	1F (0.9)	3B,C,D (3.2)	8/1511 (0.52)
Litter incidence (%)	1 (5.56)	1 (8.3)	1 (6.67)	3 (23.1)	8/188 (4.25)
Anomalies					--
Total number of foetuses examined#	160	101	107	89	--
Number of foetuses with gross/visceral anomalies (%)	9 (6.4)	14 (19.5)	14 (12.9)	6 (9.6 (K))	--
Number of foetuses with skeletal anomalies (%)	21 (11.7)	13 (17.7)	14 (12.5)	11 (10.1 (K))	--
Number of foetuses with reduced ossification (%)	7 (4.4)	4 (4.0)	5 (4.7)	4(4.5)	--
Mean foetal weight of foetuses with reduced ossification (g)	37.9	43.6	37.7	26.1	--

◊ Number affected / total number examined

Malformed foetuses are excluded

* Retroesophageal right subclavian artery is considered a variation by other laboratories (Solecki *et al.*, 2014)

(F) Fisher's exact test applied, not statistically significant (p > 0.05)

(K) Kruskal-Wallis 'H' statistic, not significant (p > 0.05)

-- no data

A,B,C,D,E,F,G, H, I, J, K - Represents different foetuses

The study CA 5.6.2/016 (1989), regarded as not acceptable in the RAR B-6.6 and performed with NZW rabbits, was described to have several serious reporting deficiencies, including no individual data, no statistical analysis, no uterine weights and no results from maternal necropsy. Further, no HCD were included in the study report. Maternal toxicity was reported in the high dose group as lower food consumption and reduced bw gain. In this study the total number of foetuses and litters with malformations were higher at 250 and 500 mg/kg bw/d relative to controls (3 foetuses (3 litters), 6(6), 10(10) and 20(12) from the 0, 125, 250 and 500 mg/kg bw/d dose groups, respectively) and included ventricular septal defects (0, 1(1), 1(1) and 2(2) foetuses (litters) from the 0, 125, 250 and 500 mg/kg bw/d dose groups, respectively). Other malformations included abnormal tail (foetal (litter) incidence of 1(1), 1(1), 2(2) and 3(2)), absent kidney(s) (foetal (litter) incidence of 1(1), 2(2), 2(2) and 6(4)), absent post-caval lung lobe (foetal (litter) incidence of (0, 1(1), 2(2) and 4(3)) and rudimentary 14th rib (foetal (litter) incidence of 1(1), 0, 2(2) and 5(2)). No information regarding statistical significance was included in the study. It is not clear form the study reporting whether the different malformations were found in different foetuses or if some foetuses had multiple malformations. In the high dose, the total number of litters with malformations was 12. However, the number of animals on the study was 15 and out of these three were reported as being non-pregnant and two as having aborted. Despite, the number of litters examined is reported to be 12 in the high dose group which implies that aborted foetuses were examined and that data from these two litters were included in the analysis. RAC concludes that due to serious reporting deficiencies in the study the results from this study should be treated with great caution.

The developmental toxicity study CA 5.6.2/011 (1995), considered acceptable in the RAR B-6.6, was performed with Japanese white rabbits with doses of glyphosate at 0, 10, 100 and 300 mg/kg bw/d. In this study a statistically significant increase in the numbers of litters with skeletal malformations were reported. The litter/foetus incidences were 1/1 (5.5/0.7%), 3/4 (20/3.1%), 3/6 (18.8/4%) and 5/5 (35.7/4.5%) in the 0, 10, 100 and 300 mg/kg bw/d dose groups, respectively. The most frequent malformations were fissure (0, 0, 3 and 0 foetuses in the low, mid and high dose group, respectively) or splitting (0, 0, 3 and 1 foetuses in the low, mid and high dose group, respectively) of the parietal bones. In the low and high dose groups, 1 foetus and 2 foetuses had fusion of parietal bones. The impact of the increase in skeletal malformations was difficult to interpret since a litter is counted whether only one or all foetuses are affected, and for most of the skeletal malformations 1-2 foetuses/litter were affected. Visceral malformations were reported in one foetus at 10 mg/kg bw/d (fusion of the right pulmonary lobe and dilatation of the lateral ventricles). At 100 mg/kg bw/d, two foetuses from the same litter had fusion of the right pulmonary lobe and one of the foetuses also had undescended testis. One foetus from another litter had hypoplasia of the pulmonary arteria with ventricular septal defects. However, it is noted that no similar effect on the craniofacial skeleton was recorded in the other acceptable rabbit studies at dose levels up to and including 500 mg/kg bw/d. The maternal toxicity reported included one maternal death in the high dose group, abortions (2 in low and 2 in high dose group) and loose stool. No effects were reported on food intake or body weight. RAC concludes that the skeletal craniofacial malformations reported at low incidences in one study but not found in the other six rabbit developmental toxicity studies were considered to be anomalous and were given less weight in the overall weight of evidence.

The developmental toxicity study CA 5.6.2/019 (1980, supportive in RAR B-6.6) was performed with Dutch belted rabbits with doses of glyphosate at 0, 75, 175 or 350 mg/kg bw/d. In this study the number of foetuses (litters) with malformations were 0, 3(3), 2(2) and 2(1) from the 0, 75, 175 and 375 mg/kg bw/d dose groups, respectively. Soft tissue malformations were reported in two foetuses in the high dose group (one with carpal flexure and one with gastro-thoracoschisis and foetal anasarca). Skeletal malformations were reported in the low and mid dose groups (anencephaly, absent rib, malformed rib and fused cervical vertebral centre). The

maternal toxicity reported included maternal death (0, 1, 2 and 10 in the 0, 75, 175 and 350 mg/kg bw/d dose groups, respectively), soft stool and diarrhoea. No effects on maternal body weight and body weight gain were reported. RAC considers that the high incidence of maternal deaths (10 female rabbits died) in the high dose group leads to an insufficient number of litters being available for assessing possible adverse effects on foetal development at 375 mg/kg bw/d in this study.

In summary, the increases in interventricular septal defects in the study CA 5.6.2/014 (1991) the increase in ventricular septal defects in the study CA 5.6.2/016 (1989) and the increase in the incidence of dilated heart in the study CA 5.6.2/012-013 (1993) may give some concern for the induction of visceral malformations in the heart following *in utero* exposure to glyphosate in rabbits. However, CA 5.6.2/016 (1989) was reported to have serious deficiencies and considered as not acceptable. In the studies CA 5.6.2/012-013 (1993) and CA 5.6.2/019 (1980), high maternal death was reported in the high dose group (500 mg/kg bw/d and 350 mg/kg bw/d) leading to insufficient number of foetuses being available for assessment. Furthermore, the cardiovascular malformation related to treatment with glyphosate was not reported consistently in the seven developmental toxicity studies in rabbits, and when reported the incidences were low and without clear dose-response relationship and were also reported in the control groups. An increase in cranial bone malformations (fissure and or splitting of parietal bones) was reported in the study CA 5.6.2/011 (1995). However, no similar finding was reported in the other acceptable studies in rabbits.

Human information

The DS did not include human data in their evaluation of effects on development. There are however several epidemiological studies available in the open literature reporting effects such as miscarriage, fecundity, pre-term delivery, gestational diabetes mellitus, birth weights, congenital malformations, neural tube defects, attention-deficit disorder/attention-deficit hyperactive disorder including Arbuckle *et al.* (2001), Savitz *et al.* (1997), Garry *et al.* (2002), Bell *et al.* (2001), Aris (2011) and Benítez-Leite *et al.* (2009). There are uncertainties regarding type of formulation, exposure levels, simultaneous exposure to more than one pesticide, statistically significant positive associations and the influence of recall bias, which precludes the assessment of the reliability of this information and the data are not considered to establish a clear link between exposure to glyphosate and developmental toxicity.

In addition, a study by Eaton *et al.* (2022) was included during the targeted consultation of glyphosate and studied urinary biomarkers of lipid oxidative stress in 347 urine samples collected between 16-20 weeks, and 24-28 weeks of gestation from pregnant women in the PROTECT birth cohort from Puerto Rico. Glyphosate and AMPA were measured in urine as well as three biomarkers of oxidative stress: 8-isoprostane-prostaglandin-F2α (8-iso-PGF2α), its metabolite 2,3-dinor-5,6-dihy dro-15-F2 t-isoprostane (8-isoprostane metabolite) and prostaglandin-F2α (PGF2α). The association between exposures and oxidative stress was adjusted for maternal age, smoking status, alcohol consumption, household income and specific gravity. Potential nonlinear trends were also assessed using tertiles of glyphosate and AMPA exposure levels. Results: the urinary geometric mean of glyphosate was 0.49 ng/mL and for AMPA 0.30 ng/mL. The association with increased levels of lipid oxidative stress biomarkers were strongest for samples collected at 24 - 28 weeks of gestation, while samples collected at 16 - 20 weeks of gestation were close to the null. An interquartile range increase in AMPA was associated with 9.5% higher 8-iso-PGF2α metabolite concentrations (95% CI: 0.5 - 19.3%). In addition, when comparing the lowest exposure group with the second and third tertiles of AMPA, significantly association with 12.8% (0.6 - 26.5%) and 15.2% (1.8 - 30.3%) higher 8-isoprostane metabolite was seen, respectively. The study concluded that urinary concentrations of AMPA, were associated with higher levels of certain oxidative stress biomarkers, and that glyphosate showed similar trend, but not as marked

as for AMPA. RAC notes the small sample size in the study, and that AMPA is also a degradation product of other substances (e.g., amino-polyphosphates) making it difficult to distinguish the proportion of AMPA present as a result of glyphosate exposure.

In summary, there is no convincing evidence of developmental effects following *in utero* exposure to glyphosate from epidemiological studies.

Comparison with the CLP criteria

Repr. 1A

There are no clear indications of effects on development following exposure of glyphosate to humans, therefore RAC considers that classification of glyphosate as Repr. 1A is not justified.

Repr. 1B

According to the CLP criteria, classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on development in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on development is considered not to be a secondary non-specific consequence of other toxic effects and for Repr. 2.

Repr. 2

According to the CLP criteria, a classification of a substance in Category 2 is justified when there is some evidence from humans or experimental animal, possible supplemented with other information of an adverse effect on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification.

In five developmental toxicity studies performed in rats, no consistent adverse effects were reported on development and RAC considers that classification for developmental toxicity is not justified based on these studies.

In the eight developmental toxicity studies performed in rabbits (4 acceptable, 1 supplementary, 1 supportive and 1 pilot study), some evidence of adverse effects on development were observed in five of the studies (all performed in different laboratories, four described as acceptable in the RAR B-6.6) at dosage levels far lower than those used in the rat studies and thus indicating that pregnant rabbits are a more sensitive species than the pregnant rat following oral exposure to glyphosate. The developmental toxicity reported included statistically significant increases in late embryo-foetal death, post-implantation loss as well as skeletal and visceral malformations, although at low incidences, which for some of the effects was without a clear dose-response relationship and not consistently reported in all eight rabbit developmental toxicity studies.

Post-implantation loss and late/early embryo-foetal death were reported in only two (acceptable) out of the seven rabbit studies. Based on the weight of evidence assessment RAC concludes that following *in utero* exposure to glyphosate in rabbits no clear relationship between exposure and effects on foetal viability could be determined. Effects on foetal viability were not reported consistently in the four acceptable developmental toxicity studies in rabbits. Only one study (CA 5.6.2/014, 1991) reported effects on foetal viability, however, without a clear dose-response relationship and within the historical control range for late and total embryonic deaths.

Visceral and skeletal malformations were reported in five (three acceptable) out of the eight rabbit studies. Based on the weight of the evidence, RAC concludes that the reported increases in visceral malformations including interventricular septal defects in the study CA 5.6.2/014 (1991), the increase in ventricular septal defects in the study CA 5.6.2/016 (1989), and the

increase in dilated heart in the study CA 5.6.2/012-013 (1993) give some evidence that cardiovascular malformations in the heart can be induced following *in utero* exposure to glyphosate in rabbits. The studies CA 5.6.2/016 (1989) and CA 5.6.2/012-013 (1993) were reported to have serious deficiencies. In the study CA 5.6.2/012-013 (1993) and CA 5.6.2/019 (1980) high maternal death was reported in the high dose group (500 and 350 mg/kg bw/d, respectively) leading to insufficient number of foetuses being available for assessment. The cardiovascular malformations related to treatment to glyphosate were not reported consistently in the eight developmental toxicity studies in rabbits, and when reported, the incidences were low, without a clear dose-response relationship and were also reported in the control groups. Skeletal malformations were reported in the study CA 5.6.2/011 (1995); however, a statistically significant increase in skeletal craniofacial malformations were not seen in the other acceptable rabbit developmental toxicity studies.

In conclusion, the five studies with rats with doses up to 3500 mg/kg bw/d showed insufficient evidence of developmental toxicity following *in utero* exposure to glyphosate including reduced ossification and skeletal malformations at maternally toxic doses, with a LOAEL for developmental effects ≥ 1000 mg/kg bw/d.

In the eight developmental toxicity studies in rabbits, limited evidence of cardiovascular malformations, skeletal malformations, post-implantation loss and embryo-foetal death were reported following *in utero* exposure to glyphosate since no clear picture of these effects were reported across the four acceptable rabbit developmental toxicity studies. These effects were reported at low incidences, and in some of the studies without a clear dose-response relationship. Further, it should be noted that the cardiovascular malformations were to some extent clustered together in the same foetuses. Skeletal malformations evident as craniofacial malformations were reported in one study (CA 5.6.2/011, 1995) however, it is noted that no similar malformations were recorded in the other seven acceptable studies at dose levels up to and including 500 mg/kg bw/d. The effects were reported in the presence of severe maternal toxicity including death of the female rabbits and GI tract intolerance to glyphosate exposure. However, it should be kept in mind that some of the deaths were related to mis-gavage and therefore not substance related. Furthermore, in some of the studies serious deficiencies in the reporting of the results were evident.

Epidemiological studies show no convincing evidence of developmental effects following *in utero* exposure to glyphosate.

Overall, in a weight of evidence assessment **RAC concludes that no classification for adverse effects on development is warranted**.

Adverse effects on or via lactation

There are no specific studies submitted for effects on or via lactation. Further, it is noted that adverse effects on or via lactation was not assessed in the 2017 RAC opinion.

In the reproductive toxicity generational studies, reduced pup weight was observed in individual studies, however at limit dose level (1000 mg/kg bw/d) and above. Delayed sexual maturation (preputial separation) was observed at limit test dose (1000 mg/kg bw/d), and distended caecum was observed at the very high dose of 2000 mg/kg bw/d.

Further, studies from the open literature, which were considered reliable with restrictions, did not provide evidence relevant for a classification for adverse effects on or via lactation.

Comparison with the CLP criteria

There are no human evidence indicating a hazard to babies during the lactation period. Further the available one or two generation reproductive toxicity studies in animals does not provide clear evidence of adverse effect in the offspring due to transfer in the milk or adverse effect on the quality of the milk. Finally, there are no data available from absorption, metabolism, distribution and excretion studies that indicate that the substance is present in potentially toxic levels in breast milk. RAC concluded that **no classification for adverse effects on or via lactation is warranted**.

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

The DS noted that according to the CLP Regulation, classification for aspiration hazard relates to liquids or mixtures only, and since the substance is a solid (and no data were available), no classification was proposed.

Comments received during consultation

No comments were submitted on aspiration toxicity during the consultation.

Assessment and comparison with the classification criteria

RAC has not evaluated aspiration toxicity.

ENVIRONMENTAL HAZARD EVALUATION

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

Glyphosate has a harmonised classification as Aquatic Chronic 2; H411 in Annex VI of the CLP Regulation. The DS proposed **to retain the classification** based on the substance being not rapidly degradable and having a low potential for bioaccumulation. The lowest reliable acute aquatic toxicity value was the 72h EC_{50} of 13.5 mg acid equivalent/L for *Skeletonema costatum*. As this value was greater than the 1 mg/L CLP cut-off the criteria for acute aquatic classification were not fulfilled. The lowest reliable aquatic chronic value was a 7d NOEC of 1 mg /L for *Danio rerio.* With Glyphosate being not rapidly degradable, an Aquatic Chronic 2; H411 classification was considered warranted by the DS.

Degradation

Table: *Summary of relevant information on rapid degradability (Key studies)*

Method	Test substance	Results	Reference
Ready biodegradability OECD TG 301F	Glyphosate, 97.7%	Biodegradation after 28d was 26%, not readily biodegradable	CA 7.2.2.1/001, 2009
Hydrolysis BBA-Merkblatt No. 55, part I and II (October 1980)	Glyphosate isopropylamine salt, 98%	Glyphosate was stable to hydrolysis at pH 5, 7 and 9 at 23 and 50°C (< 10% after 29d)	CA 7.2.1.1/004, 1993
Hydrolysis OECD TG 111	Glyphosate monosodium salt, 97.5%	Glyphosate was stable to hydrolysis at pH 4, 7 and 9 at 50°C (< 10% after 5d)	CA 7.2.1.1/005, 1992
Hydrolysis US EPA 540/9-85-013: section 161-1	^{14}C-Glyphosate (PMG), 97.4%	Glyphosate was stable to hydrolysis at pH 5, 7 and 9 at 25°C (no hydrolysis products observed after 30d)	CA 7.2.1.1/007, 1990
Aerobic mineralisation OECD TG 309	^{14}C-Glyphosate (PMG), 98.3%	DT_{50}: 12.3 and 21.8 days at low and high dose, respectively. Mineralisation: 23.1 - 26.5% AR after 62 days	CA 7.2.2.2/001, 2020
Degradation in water/sediment systems BBA Guideline Part IV, 5-1 SETAC 1995	Glyphosyte-Trimesium, 14C-PMG labelled, > 99%	After 100d, glyphosate, % AR: <u>Water</u> Cache: 0.8 Putah: 5.1 <u>Sediment</u> Cache: 3.7 Putah: 58.2 <u>Total system</u> Cache: 4.5 Putah: 63.3 Mineralisation after 100d, % AR: Cache: 48.0 Putah: 5.9 Non-extractable residues after 100d, % AR: Cache: 13.5 Putah: 16.7 <u>Total system</u>, $DegT_{50}$, days: Cache: 8.4 Putah: 195.8 <u>Water</u>, $DisT_{50}$, days Cache: 5.0 Putah: 7.9 <u>Sediment</u>, $DisT_{50}$, days Cache: 33.9 Putah: not derived	CA 7.2.2.3/002, 1999 / Kinetic analysis in 2020
Degradation in water/sediment systems BBA Guideline Part IV, 5-1	^{14}C Glyphosate, PMG labelled, 98.9% (HPLC)	After 100d, glyphosate, % AR: <u>Water</u> Bickenbach (B) 0.3 Unter Widdersheim (U) 2.4 <u>Sediment</u> B: 29.2 U: 44.1 <u>Total system</u> B: 29.5 U: 46.6 Mineralisation after 100 days, % AR: B: 23.5 U: 17.8 Non-extractable residues after 100d, % AR: B: 22.0 U: 13.6 <u>Total system</u> $DegT_{50}$, days: B: 15.8 U: 121.6	CA 7.2.2.3/005, 1993, Amendment 1995/ Kinetic analysis in 2020

Method	Test substance	Results	Reference
		<u>Water</u> DisT$_{50}$, days: B: 2.0 U: 1.1tokyo <u>Sediment</u> DisT$_{50}$, days: B: 158.7 days U: Not derived.	
Inherent biodegradation, OECD TG 302B	Glyphosate, 97.7%	Glyphosate was not inherently biodegradable (2% after 28d)	CA 7.2.2.1/002, 1991
Inherent biodegradation, OECD TG 302B	Glyphosate, 98.9%	Glyphosate was not inherently biodegradable (0% after 28d)	CA 7.2.2.1/003, 1990

PMG=Glyphosate N-(phosphonomethyl)glycine

Photolysis: three studies were available. In the first study, the photoinduced degradation half-life of glyphosate in natural water ranged from 33.9 to 34.4 days (Tokyo, spring) based on pseudo-first order kinetics (indirect photolysis). The major degradation products detected were AMPA and methanediol. No degradation of glyphosate was observed in distilled water. In the second study in artificial light, degradation of glyphosate was slightly enhanced under irradiated conditions. The photolytic degradation DT$_{50}$ values in aqueous solutions were 33, 69 and 77 days at pH 5.1, 7.3 and 9.2, respectively (direct photolysis). AMPA was detected as a degradation product. In the third study in buffer solutions exposed to natural sunlight up to 31 days, no significant degradation of glyphosate was observed at pH 5, 7 and 9 (direct photolysis).

Ready biodegradability: in the only available test (OECD TG 301F) only 26% of glyphosate degraded after 28 days indicating that it was not readily biodegradable.

Hydrolysis: glyphosate was stable at pH 4-5, 7 and 9 in the three available hydrolysis studies. More than 90% of the applied active substance remained at the end of the study.

Aerobic mineralisation in a surface water system: in the one available study, the mineralisation of glyphosate was studied at two concentrations, 10 µg/L and 95 µg/L containing suspended sediment, for 62 days. Dissipation of glyphosate in the surface water system occurred through a combination of microbial degradation and formation of non-extractable residues in the suspended sediment. The only metabolite identified in the water phase was AMPA. The maximum mineralisation of glyphosate achieved was 26.5 and 23.1% of applied radioactivity (AR), while non-extractable residues reached a mean maximum level of 14.0 and 9.1% in the low and the high dose, respectively. The dissipation rate of glyphosate in the total system was evaluated using a single first order kinetic model giving DT$_{50}$ values of 12.3 and 21.8 days for the low and high dose, respectively.

Degradation in water/sediment system: two studies were available. In study CA 7.2.2.3/002, 1999, glyphosate was tested in two water sediment systems (Cache, loamy sand and Putah, silt loam). Glyphosate dissipated rapidly from surface water. The rapid initial loss of glyphosate from the surface waters was most likely due to binding to the sediment. The only major metabolite detected was AMPA. Maximum amounts of carbon dioxide reached were 48% and 5.9% AR in the Cache and Putah system, respectively. In the total system, DegT$_{50}$ ranged between 8.4 - 196 days. DisT$_{50}$ ranged between 5.0 - 7.9 days in the water compartment and was 33.9 days in sediment.

In study CA 7.2.2.3/005, 1993/1995, glyphosate was tested in two water sediment systems (Bickenbach sand and Unter Widdersheim loam). AMPA and HMPA (hydroxymethylphosphonic acid) were identified in the water phase of both test systems. Maximum amounts of carbon dioxide were 23.48% AR at 100 day after treatment and 19.37% AR at 61 days in Bickenbach and Unter Widdersheim systems, respectively. In the total system, DegT$_{50}$ ranged between 15.8

- 121.6 days. DisT$_{50}$ ranged between 1.1 - 2.0 days in the water compartment and was 158.7 days in sediment.

Inherent biodegradability: in the two available tests (both according to OECD TG 302B), biodegradation after 28 days was 2% and 0%, respectively, showing that glyphosate was not inherently biodegradable.

<u>Conclusion</u>

The DS concluded that based on the substance being not readily or inherently biodegradable and not degrading in the aquatic environment to a level of 70% or above within 28 days in hydrolysis or water/sediment studies, glyphosate is considered not rapidly degradable.

Bioaccumulation

Table: *Summary of relevant information on bioaccumulation*

Method	Test substance	Results	Reference
US EPA Guideline 72-6 Supportive study *Lepomis macrochirus*	[14]Glyphosate (N-phosphonomethylglycine-methyl-^{14}C), 99.2%	BCF value not reliable Indication of low bioaccumulation potential	CA 8.2.2.3/001, 1989 CA 8.2.2.3/002, 1989
OECD TG 107 (shake flask method) OPPTS 830.7550 GLP	Glyphosate, 99.9%	Log K_{ow} at 25 °C: -5.39 (pH 5) -6.28 (pH 7) -5.83 (pH 9)	Report no. 139K-101 CA 2.7/001, 2020a
OECD TG 107 (shake flask method) GLP	Glyphosate, 99.5%	Log K_{ow} at 20°C: < 3.4	Report no. 238498 CA 2.7/002, 1990
OECD TG 107; EEC A.8 OPPTS 830.7550 GLP	Glyphosate K-salt, 91.8%	Log K_{ow} at 20°C: < -0.7 (pH 3.16)	Report no. 497741 CA 2.7/006, 2012
OECD TG 107 OPPTS 830.7550 GLP	N-acetyl glyphosate, 93%	Log K_{ow} at 25°C: -6.29 (pH 5) -6.26 (pH 7) -6.86 (pH 9)	Report no. 139K-104 CA 2.7/008, 2020b

In the only available (US EPA guideline OPP 72-6) study on bioconcentration potential (CA 8.2.2.3/001, 1989), bluegill sunfish (*Lepomis macrochirus*) was exposed to ^{14}C-glyphosate for 35 days. Flow-through was used to maintain a mean measured water concentration of 12 ± 0.7 mg/L. Subsequently, the fish were exposed for 21 days to flowing uncontaminated well water. The daily bioconcentration factor ranged from < 0.11 to 0.52 for whole fish. Uptake tissue concentrations of ^{14}C-glyphosate ranged from < 1.3 to 6.2 mg/kg for whole fish. ^{14}C-residue levels were below minimum quantifiable limits until day 21 for fillet and day 7 for whole fish and viscera samples. Water samples from treatment days 1, 28, and 35 of the bioconcentration phase were analysed by HPLC and found to contain 95 - 97% glyphosate with 1.1 - 1.9% AMPA.

The time to reach 90% of steady state was estimated to be 120 ± 59 days but the steady state was not achieved during 35 days of uptake. The bioconcentration factor (BCF) was estimated to be 1.1 ± 0.61.

However, the test was conducted long before the most recent revision of OECD TG 305. Since then, experience has shown that biological factors such as growth and fish lipid content needs to be taken into account. In the available study, fish lipid content was not measured and kinetic BCFs may not have been corrected for growth dilution. In addition, a steady state could not be observed because concentrations in fish were still increasing at the end of the uptake phase. Other differences compared to the OECD TG 305 were: no use of reference substance, only one

concentration tested although glyphosate is a polar compound, and the test concentration might have been too high.

Overall, the DS considered that the study was not robust enough to derive a BCF value. Even if no reliable numerical BCF value could be set, the overall outcome indicated a low potential for bioaccumulation.

There were four reliable OECD TG 107 studies available giving log K_{ow} values from – 6.86 to < -0.7. These results also showed low potential for bioaccumulation.

Aquatic toxicity

The CLH dossier has been prepared as a part of the RAR according to the PPP Regulation. The RAR/CLH dossier contains numerous studies for the aquatic compartment:

- Fish: 18 short-term and 9 long-term studies,
- Aquatic invertebrates: 18 short-term and 10 long-term studies,
- Algae: 20 studies with acute and chronic endpoints,
- Aquatic plants: 7 studies with acute and chronic endpoints,
- Other aquatic organisms: 1 short-term and 1 long-term study (amphibians).

In addition to these, the CLH dossier includes additional toxicity studies for amphibians (Vol. 1, 2.9.1.6).

The CLH dossier also presents studies for formulations containing glyphosate and for glyphosate metabolites.

Acute aquatic toxicity

A summary of the relevant information on acute aquatic toxicity can be found in table 70 of the RAR/CLH dossier.

Table: *Summary of the lowest reliable acute toxicity values in the RAR/CLH dossier*

Method	Species	Test material	Results	Remarks	Reference
Fish					
US EPA Guideline, FIFRA subdivision E, section 71-1 GLP	*Lepomis macrochirus*	Glyphosate, 95.6%	LC$_{50}$ (96h): > 32 mg/L (nom)	Valid with restrictions: pH outside the recommended range at all tested concentration. Endpoints set at the highest dose without mortality	CA 8.2.1/009, 1995a
Aquatic invertebrates					
No guideline followed Non-GLP Literature data	*Crassostrea gigas*	Glyphosate, 97%	LC$_{50}$ (48h) > 100 mg/L EC$_{50}$ (48h)=27.1 mg/L	Reliable	Mottier A. *et al.*, 2013 (CA 8.2.8) Literature data
Algae					
OECD TG 201 (1984) US EPA Guideline 540/09-82-020 (1982) GLP	*Skeletonema costatum*	Glyphosate, 95.6%	**72h E$_r$C$_{50}$=13.5 mg/L (nom)**	Valid	CA 8.2.6.2/006, 1996a, Report no. AB0503/I CA 8.2.6.2/007, 2020a, Report no. 110054-007 (updated statistical evaluation)

Aquatic plants					
OECD TG 221 GLP	*Lemna minor*	Glyphosate isopropylamine salt, 97.1%	Frond number 7d E_rC_{50}=30.3 mg a.e./L (nom) Phytotoxicity NOEC=8.65 mg a.e./L (nom)	Valid Results based on statistical re-evaluation	CA 8.2.7/001, 2002, Report no. CEMR-1873 CA 8.2.7/002, 2020f, Report no. 110054-008 (updated statistical evaluation)
Other aquatic organisms					
OECD TG 241; ASTM E1439-12 Non-GLP	*Physalaemus cuvieri* (tadpoles Gs 25)	Glyphosate, 99.2%	96h LC_{50}=115 mg/L	Reliable with restrictions. Literature article. Validity criteria not reported. No analytical test item verifications	Daam *et al.*, 2019, CA 8.2.8/001

a.e.=acid equivalent

Summary of short-term fish studies

For acute toxicity in fish, eighteen studies were available from different species (i.e. *Lepomis macrochirus*, bluegill; *Oncorhynchus mykiss;* rainbow trout; *Cyprinus carpio*; common carp; *Leuciscus idus,* golden orfe; *Danio rerio,* zebrafidh). Three studies (CA 8.2.1/014, 1973; CA 8.2.1/003, 1995a; CA 8.2.1/008, 1972, in CLH dossier) lacked a detailed report and were thus excluded from further evaluation. One study (CA 8.2.1/011, 1981b) was considered invalid due to serious drawbacks including the absence of analytical verification of glyphosate exposure and major deviations from the validity criteria. Six studies were considered relevant and reliable (Klimisch score 1-2), while eight studies were regarded as supportive due to limitations in the experimental design, ranging from the use of individuals with larger size than recommended, the absence of Good Laboratory Practise (GLP) and the use of species not listed in the OECD TG 203.

The relevant and valid studies were conducted according to US-EPA or OECD TG 203 and were compliant with GLP. Glyphosate was tested at various degrees of purity (ranging from 98.9% to 47.7%) and different chemical forms, including technical glyphosate, glyphosate potassium salt and glyphosate isopropylamine salt. All the relevant studies fulfilled the validity criteria of their respective guidelines.

The most acutely sensitive fish species was *Lepomis macrochirus* (CA 8.2.1/009, 1995a, study valid with restrictions) which was exposed to nominal test concentrations of 10, 18, 32, 56, 100 and 180 mg/L glyphosate under static conditions for 96 hours. Measured concentrations remained within 80 and 120% of nominal and the ecotoxicological endpoints were evaluated using nominal concentrations. The reported 96h LC_{50} was 47 mg/L, with a 95% CI of 35 to 66 mg/L. At glyphosate concentrations of 10, 18 and 32 mg/L, no mortality was recorded while pH values far below the recommended range (6.5 - 8.5) in the OECD TG 203 were measured at the three highest concentrations of 56, 100, 180 mg/L. In the absence of the appropriate controls, it cannot be excluded that the severe toxicity observed at these high concentrations was actually caused by the acidification of the test medium.

Summary of short-term invertebrate studies

A total of eighteen studies have been surveyed by the DS to assess acute toxicity of glyphosate in aquatic invertebrates of which, fourteen studies have been used for classification purposes. Most of the selected studies were conducted in compliance with GLP and in agreement with internationally accepted methods (i.e., OECD, EPA FIFRA or ASTM testing guidelines). Two studies were not performed according to GLP or official protocols but were selected from the literature based on scientific quality and reliability. According to the fulfilment of

validity/reliability criteria, six studies have been categorised as valid/reliable, seven as valid/reliable with restrictions and one as supportive. Data include endpoints measured in crustaceans (ten studies), molluscs (three studies) and cnidarians (one study); median effective (or lethal) concentrations measured in valid/reliable studies range from 27.1 to > 471 mg/L. The lowest LC_{50} value of 27.1 mg/L was derived in a literature study by Mottier *et al*. (2013) assessing the 48h embryotoxicity of glyphosate in the pacific oyster (*Crassostrea gigas*) according to the ISO guideline no. 17244:2015 at nominal test concentrations comprised between 0.0001 and 100 mg/L. The validity criteria according to the test guideline were fulfilled.

Summary of short-term algae studies

20 studies with acute effects are presented in the CLH dossier

The study that presented the lowest EC_{50} value is on *Skeletonema costatum* according to OECD TG 201 (CA 8.2.6.2/006, 1996a).

The effects of glyphosate (purity 95.6%) on *Skeletonema costatum* were evaluated in a 120h, static test. The test incorporated 8 nominal concentrations of glyphosate (1.0, 1.8, 3.2, 5.6, 10, 18, 32, and 56 mg/L), and a control consisting of culture medium without test item.

The calculated 72h EC_{50} value was 13.5 mg/L for growth rate. All validity criteria were met according to guideline OECD TG 201: this study is considered valid and reliable for classification purposes.

Summary of short-term aquatic plant studies

Seven studies on the acute and chronic effects on aquatic plants were assessed. Three of these were considered valid and the remaining four invalid, due to: non-fulfilment of the validity criteria (CA 8.2.7/010, 2012), uncertainties on the exposure (CA 8.2.7/003, 1999; statistical re-analysis in CA 8.2.7/004, 2020) or because the report was not available (CA 8.2.7/013, 2015; CA 8.2.7/009, 1987).

All three valid studies were conducted according to Guideline 123-2, US EPA FIFRA or OECD TG 221 and under GLP on duckweed species (*Lemna minor* and *Lemna gibba*). The acute effects ranged from 16.5 to above 49.4 mg/L. The test items were glyphosate isopropylamine salt (IPA-salt) and glyphosate technical. All three relevant studies fulfilled the validity criteria and underwent a statistical reanalysis.

The lowest acute values were obtained in a study conducted in 2002 (CA 8.2.7/001, updated statistical evaluation in CA 8.2.7/002, 2020). The study followed the OECD TG 221 and tested the effect of glyphosate IPA-salt on the growth of *Lemna minor* in a 7d semi-static toxicity test at nominal concentrations of 2.92, 5.83, 11.7, 24.3, 48.6 and 97.2 mg/L. The mean measured concentrations ranged between 96 and 104% of the nominals. The 7d E_rC_{50} was 30.3 mg a.e./L (nominal) based on frond numbers.

<u>*Summary of short-term amphibian studies*</u>

The Daam *et al.*, 2019 study conducted partially according to OECD TG 241 investigated the acute toxicity of glyphosate to tadpoles of two tropical frog species *Physalaemus cuvieri* and *Hypsiboas pardalis*. The nominal test concentrations were 84, 97, 112, 130 and 150 mg/L. The calculated LC_{50} values for *P. cuvieri* and *H. pardalis* were 115 and 106 mg/L, respectively. However, validity criteria were not reported. It was unknown if the larvae were exposed to any other chemicals, as no analysis of watershed water was provided. There was no analytical verification of test concentrations reported. The study was considered by the DS as reliable with restrictions.

In the Turhan *et al.,*2020 study (CA 8.1.4 in CLH dossier), effects of pure glyphosate were evaluated using two embryonic development stages of *Xenopus laevis* as a model system (embryos and stage 46 tadpoles). No lethal or developmental effects were observed at all concentrations tested. The 96h LC_{50} values for glyphosate could not be determined due to low mortality among both embryos and tadpoles (i.e., max 17% mortality). Thus, the LC_{50} was above 403 mg/L for tadpoles and > 500 mg/L for embryos. Measured biological parameters included growth (length) and measuring enzyme levels. The study was considered reliable with restrictions by the DS, since analytical verification was only performed on test solutions from the 'FETAX' test, not the tadpole toxicity bioassays.

The Bach *et al.*, 2016 study (CA 8.1.4 in CLH dossier) investigated the effects on the growth, development and induction of abnormalities of glyphosate active ingredient during two developmental stages of the South-American Creole frog, *Leptodactylus latrans*. Test concentrations ranged from 3 to 300 mg/L. Chemical analysis was performed showing that concentrations remained constant, with no lethal effects observed. The LOEC for development and growth of Gosner stage (Gs) 25 larvae were both 15 mg/L, while the LOEC for morphological abnormalities was 30 mg/L for both Gs 25 and Gs 36.

DS conclusion on acute aquatic toxicity

The DS concluded that the lowest reliable acute endpoint for glyphosate was the 72h EC_{50} of 13.5 mg /L for *Skeletonema costatum.* Based on all this information, the DS concluded that acute classification was not warranted.

Chronic aquatic toxicity

A summary of the relevant information on chronic aquatic toxicity can be found in Table 71 of the RAR/CLH dossier.

Table: Summary of the lowest reliable chronic toxicity values in the CLH dossier

Method	Species	Test material	Results	Remarks	Reference
Fish					
IBAMA 1990: Manual de testes para avaliacao da ecotoxicidade de agentes quimicos GLP	*Danio rerio*	Glyphosate, 95.5%	**NOEC (7 d)=1 mg/L (nom)**	Valid	CA 8.2.2.1/002, 2000
Aquatic invertebrates					
OECD TG 202, Part II, Reproduction Test (1984) GLP	*Daphnia magna*	Glyphosate, 97.6%	NOEC (21 d): 12.5 mg/L (nom)	Valid-	CA 8.2.5.1/001, 1999, Report no. AF0497/B
Algae					
OECD TG 201 (1984) US EPA Guideline 540/09-82-020 (1982) GLP	*Skeletonema costatum*	Glyphosate, 95.6%	72h NOE_rC=5.6 mg/L 72h E_rC_{10}=1.87 mg/L 72h E_rC_{20}=2.98 mg/L (nom)	Valid	CA 8.2.6.2/006, 1996a, Report no. AB0503/I CA 8.2.6.2/007, 2020, Report no. 110054-007 (updated statistical evaluation)
Aquatic plants					
OECD TG 221 GLP	*Lemna minor*	Glyphosate isopropylamine salt, 97.1%	Frond number 7d NOE_rC=8.65 mg a.e./L (nom)	Valid Results based on statistical re-evaluation	CA 8.2.7/001, 2002, Report no. CEMR-1873

			7d E_rC_{10}=8.16 mg a.e./L (nom) 7d E_rC_{20}=12.8 mg a.e./L (nom) Phytotoxicity NOEC=8.65 mg a.e./L (nom)		CA 8.2.7/002, 2020, Report no. 110054-008 (updated statistical evaluation)
Other aquatic organisms					
OECD TG 231 (2009) OPPTS/OCSPP Guideline 890.1100 (2009) GLP	*Xenopus laevis*	Glyphosate, 85.14%	NOEC (21d) ≥ 90 mg/L (mean measured)	Valid	CA 8.2.3/002, 2012b, Report no. 707A-103

a.e.=acid equivalent

Summary of long-term fish studies

The CLH dossier contains several long-term toxicity studies on glyphosate, conducted in different fish species (i.e., *Oncorhynchus mykiss*, *Pimephales promelas and Danio rerio*). From the analysis of the 9 studies, one was considered not reliable by the DS due to the low number of replicates used (see further below). Only those studies relevant for classification are presented below.

The CLH dossier contains 2 reliable and relevant studies that have previously been used for the purpose of the environmental classification.

Study CA 8.2.2.1/002, 2000c was also included in the previous CLH dossier (2016) and was regarded by the DS as the key study since it contains the lowest chronic value for the purpose of classification, corresponding to a NOEC of 1 mg/L. According to the DS, this study was conducted following the Environmental Regulations of Brazil (IBAMA) according to the principles of GLP and can be compared to the current OECD TG 212 (Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages). The study was considered by the DS to fulfil the validity criteria of the OECD TG 212, with minor deviations.

The test was conducted for 7d under semi-static conditions (with a renewal of test media every 48h) by exposing 30 individuals for each concentration (10 for each replicate) to glyphosate concentrations ranging from 0.32 to 32 mg/L. Stock solutions containing 100 or 1000 mg/L glyphosate were freshly prepared the day of testing and their concentration was analytically measured at different intervals, up to 6 days. The analytical concentration remained in the range of 80 - 120% of nominal. The DS considered that fish mortality showed a clear dose-response relationship and that also lethargy occurred concomitantly with mortality at 3.2 mg/L, thus proposing to use a NOEC of 1 mg/L, based on both mortality and lethargy.

The second study (CA 8.2.3/001, 2012) was performed on Fathead minnow (*Pimephales promelas*) for 21 days according to OECD TG 229. The test was conducted under flow-through conditions by exposing four groups of adult males and females (2 males and 4 females in each group) to glyphosate at nominal concentrations of 0, 0.048, 0.24, 1.2, 6.0, and 30 mg/L for 21 days. Mean measured glyphosate concentrations were reported to be within the acceptance range of nominal concentrations, corresponding to 0.046, 0.23, 1.2, 6.2, and 33 mg/L, respectively. All the validity criteria of the OECD TG 229 were met, despite minor deviations in the temperature on day 7 (for less than 24 hours) that were however promptly rectified. Based on survival, fecundity, fertility and other general observations an overall NOEC of 33 mg/L was derived. The study was considered valid and reliable by the DS.

One study on Fathead minnow (*Pimephales promelas*) was considered supportive (CA 8.2.2.2/001, 1975) due to the high variability of some parameters, inconsistencies in the statistics and absence of a validated analytical method. Two other studies were not used for the purpose of classification since in one case the authors did not derive any NOEC value (Uren

Webster *et al*., 2014) while in the other case (Zhang *et al*., 2017) no standardised guidelines were used, precluding any possible conclusion on the data reliability and the latest time point considered (96h) was not sufficient for a chronic study. In a similar way, three other studies (Rodrigues *et al*., 2019; Schweizer *et al*., 2019; Gaur *et al*., 2019) were conducted according to OECD TG 236 which does not allow to derive chronic endpoints.

Study CA 8.2.2.1/001, 2010 was conducted on the Rainbow trout (*Oncorhynchus mykiss*) according to OECD TG 210 (Fish early life-stage toxicity test) under flow-through conditions, using a constant-flow delivery system in duplicate exposure vessels containing 50 fertilised eggs. The fertilised eggs (within 3.5 hours post-fertilisation (hpf)) were exposed to glyphosate nominal concentrations of 0.095, 0.305, 0.977, 3.125 and 10.0 mg/L for 85 days. The exposure concentrations were analytically determined and remained within 85.7 and 96.3% of nominal concentrations. All the validity criteria of the OECD TG 210 were met. Based on geometric mean measured concentrations, a NOEC of 9.63 mg/L was derived for survival and growth of the exposed fish. The study is considered valid yet only 2 replicates instead of 4 were used and a strong variability was observed in several endpoints. In the absence of a clear demonstration that the coefficient of variation (CV) for each response was not exceeding the 90th percentile of the CV indicated in the OECD TG 210 (table 1, Annex V), the NOEC could not be considered robust and reliable. Therefore, the DS proposed to consider the study valid but not reliable for the purpose of classification.

Summary of long-term invertebrate studies

Ten long-term studies on aquatic invertebrates (with durations ranging from 21 to 90 days) were considered suitable for chronic toxicity assessment. Eight out of ten studies were performed in compliance with GLP and/or internationally accepted methods (i.e., OECD, EPA FIFRA or ASTM testing guidelines), while the remaining two were not performed according to GLP or standardised protocols but were selected from the literature based on scientific quality and reliability criteria. All data were obtained from studies on arthropods (mainly crustaceans), with NOECs from the valid/reliable studies ranging from 12.5 to 100 mg/L. The lowest NOEC of 12.5 mg/L was derived in a 21d semi-static test performed according to OECD TG 202, Part II – Reproduction Test (1984), to assess the lethal and sub-lethal effects of glyphosate on *Daphnia magna* at nominal 12.5, 25, 50, 100, or 200 mg a. e./L (CA 8.2.5.1/001, 1999, updated statistical evaluation in CA 8.2.5.1/009, 2020). The range of measured concentrations of glyphosate in the new and old test solutions were 100 - 104% and 96 - 104%, respectively; therefore, the 21d NOEC of 12.5 mg/L was based on nominal glyphosate concentrations. All validity criteria according to the OECD TG 211 were fulfilled.

Summary of chronic algae studies

In the CLH dossier, 20 studies with chronic effects are presented.

The study that presented the lowest chronic value was on *Skeletonema costatum* according to OECD TG 201 (CA 8.2.6.2/006, 1996, updated statistical evaluation in CA 8.2.6.2/007, 2020). The effects of glyphosate (purity 95.6%) on *Skeletonema costatum* were evaluated in a static test for 120h. The test incorporated 8 nominal concentrations of glyphosate (1.0, 1.8, 3.2, 5.6, 10, 18, 32, or 56 mg/L), and a control consisting of culture medium without test item. The calculated 72h NOEC and EC_{10} values for growth rate are 5.6 and 1.87 mg/L, respectively. All validity criteria were met according to guideline OECD TG 201: this study is considered valid and reliable for classification purposes.

Summary of long-term aquatic plant studies

Seven studies were assessed for acute and chronic effects on aquatic plants. As already stated under the summary of short-term aquatic plant studies, three of them were considered valid and the remaining four invalid.

The valid studies were conducted according to US-EPA or OECD guidelines and under GLP. The chronic endpoint concentrations on duckweed species ranged from 1.5 to 31.9 mg/L. The test items were glyphosate IPA-salt and glyphosate technical. All the relevant studies fulfilled the validity criteria.

The lowest values for growth rate were obtained in a study on *Lemna minor* conduct under GLP and according to the OECD TG 221 (CA 8.2.7/001, 2002, updated statistical evaluation in CA 8.2.7/002, 2020). The effect of glyphosate IPA-salt were assessed in a 7d semi-static toxicity test at nominal concentrations of 2.92, 5.83, 11.7, 24.3, 48.6 or 97.2 mg/L. The mean measured concentrations ranged between 96 and 104% of the nominal values, therefore results were provided as nominal concentrations of acid equivalent. According to the statistical re-analysis, the 7d NOEC was 8.65 mg a.e./L for growth (frond number), for yield (dry weight and frond number) and for visual phytotoxic effects. The EC_{10} for growth based on frond number was 8.16 mg a.e./L. The EC_{10} for yield based on frond number was 8.65 mg a.e./L and based on dry weight was 5.72 mg a.e./L. Therefore, the chronic values based on growth rate reliable for the classification purposes were a 7d NOEC of 8.65 mg a.e./L and an EC_{10} of 8.16 mg a.e./L both based on frond number.

Moreover, the CLH dossier included studies on glyphosate formulations. The lowest results were obtained in a study (CP 10.2.1/006, 2012) conducted on *Myriophyllum aquaticum,* GLP compliant. The study is discussed later on this document.

Summary of long-term amphibian studies

In the only available amphibian chronic study, effects of glyphosate on amphibian metamorphosis of the African clawed frog (*Xenopus laevis*) have been investigated. The Amphibian metamorphosis assay was conducted under flow-through conditions and amphibian larvae were exposed to glyphosate at nominal concentrations of 0, 0.16, 0.80, 4.0, 20, or 100 mg/L. Arithmetic mean-measured concentrations were < 0.100, 0.13, 0.79, 4.3, 20, or 90 mg/L. There were no treatment related effects on survival, mean developmental stage, or absolute or normalized hind-limb length during the 21d test. The study was considered reliable by the DS and an overall 21d NOEC of ≥ 90 mg/L (arithmetic mean measured) was derived.

However, the DS noted that the glyphosate concentrations in the test were unstable over the time of the study especially in the highest tested treatments at 100 mg/L. There were no measurements between day 14 (106 mg/L) and day 21 (48.4 mg/L), therefore it could not be excluded that the reduced exposure of tadpoles to glyphosate in this treatment was prolonged over some days towards the end of the study.

DS conclusion on chronic toxicity

The DS concluded that the lowest reliable chronic value for glyphosate was a 7d NOEC of 1 mg/L for *D. rerio.* Based on this, and the substance being not rapidly degradable, the DS proposed to classify glyphosate to Aquatic Chronic 2.

Comments received during consultation and analysis of the studies

This section presents the comments received during the consultation via the ECHA website. Furthermore, additional information received throughout the drafting of the opinion are presented

and analysed further, as well as relevant comments received on the additional information through a targeted consultation. Part of this additional information was obtained from the review of the Environmental Quality Standard (EQS) for glyphosate. This process is in the context of the Water Framework Directive and is currently underway. A draft dossier, currently not publicly available, has been prepared by the Joint Research Centre (JRC) and was submitted to SCHEER in January 2022. The JRC has collected numerous studies for this purpose, some of which are included in the RAR, while others are not present. This section also discusses the RAC responses to these comments structured in a study-by-study basis.

Fish studies

<u>Key study CA 8.2.2.1/002, 2000</u>

The validity of the study has been challenged as reported in the following comments:

- **deviations from the IBAMA guideline** (test protocol followed for this study): according to this comment, some deviations from the IBAMA guideline have been identified, namely the use of hatched larvae instead of 24h embryos, the lack of bodyweight measurements and external feeding during the testing period that might have contributed to the mortality and lethargy at the latest time point (7d).

  RAC notes that for the purpose of the CLP Regulation, this study must be compared to the OECD TG 212 rather than to the IBAMA method, for which some requirements might not be the same as in the OECD TG 212 and, thus, any deviation would be less relevant (see table below for the full comparison of the study with OECD TG 212). For example, growth parameters were not recorded at the end of the test, therefore, effects on growth and also fish loading rates (g fish/L water) were not determined. RAC recognises that the OECD TG 212 indications suggest reporting the length and weight of the larvae in the experimental groups at the end of the exposure to account for potential effects on individuals that accidentally might have remained smaller. However, RAC notes that this is not a major drawback of the study.

  Another minor deviation refers to the fact that the holding stock tank was maintained at 28ºC while the temperature of the test media upon addition of the fish was 24.1°C. The temperature difference between the holding tank and the test tank, apparently exceeds the variability in the temperature range indicated in the OECD TG 212 (25 ± 1°C stated in Annex 3 of OECD TG 212). However, as noted by the DS, the OECD TG 212 recommends maintaining the temperature in the holding tanks at 25 ± 2°C. This temperature only slightly exceeded the recommended value and this does not seem to have affected the fish in the control group. In agreement with the DS, RAC notes that this is a minor deviation from the OECD TG 212. Furthermore, since the authors used a period of acclimation of 48h before testing and no significant mortality was seen in the control group, RAC considers unlikely that this deviation influenced the results of the test.

- the **lack of analytical measurement** of the substance concentration during the test: according to this comment, the analytical determination of glyphosate only in the stock solution up to 120h would not be sufficient to confirm the stability of the substance over the 7d period in a static system, as indicated by a slight decrease of glyphosate concentration in the stock solution at 120h. In response, the DS recognised that the lack of analytical measurements of the glyphosate concentrations at the beginning or the end of the test was a major drawback in the study.

  RAC notes that the analytical glyphosate concentration within the stock solutions did not show apparent deviations from the nominal concentrations for 6 days, so assuming that the decrease would be relevant after 7 days of exposure is questionable, when considering

the high stability of glyphosate in water. For these reasons, RAC considers it reasonable to assume that the exposure to glyphosate during the testing period remained relatively constant, despite the lack of analytical confirmation in the testing vessels themselves. RAC acknowledges that the absence of analytical measurements at the end of the test (or at the beginning of each renewal under semi-static conditions) is a potential shortcoming and does not fully allow the appropriate exposure of the fish larvae during the testing period to be confirmed. Yet, since glyphosate is stable in water and its measured concentration in the stock solutions did not show apparent deviations from the nominal concentrations, it is reasonable to assume that the glyphosate remained in the acceptable range (80 - 120% of the nominal concentration) across the test duration.

−   **media renewal/semi-static conditions** and **assessment of water quality**: according to this comment, the semi-static design of the study could not be confirmed and underlined the presence of a single set of water quality measurements in a single vessel rather than one for each renewal occasion in the freshly prepared and old media.

In this regard, RAC notes that the raw data do not allow to confirm that a static design was adopted instead of the semi-static claimed by the authors, which however is not expected to influence the outcome of the test, in this specific case. In RAC's view, the choice between static or semi-static test conditions is expected to be much more relevant in case of substances that tend to dissipate from the testing medium, either due to abiotic/biotic degradation or volatilisation, while glyphosate is a not-rapidly degradable substance with half-lives far greater than 7d and does not volatilise.

Relating to the assessment of water quality, the authors provided two types of measurements. The first concerns a single water sample wherein hardness, conductivity, pH, oxygen concentration and temperature were recorded every 24h and up to 7d. The second, more comprehensive set of measurements, is related to the continuous monitoring of the same parameters (with the exception of hardness) in each test vessel and for all the experimental conditions. For these reasons, RAC considers that the information on water quality is satisfactory.

−   the lack of information on the **age of zebrafish larvae** (and other deficiencies in the test design): according to this comment, the observed effects at later time points were actually caused by the depletion of the yolk sac followed by a further 56h of incubation without external feeding and the concomitant reduction of dissolved oxygen. In other words, there was a criticism that the study was flawed by the use of embryos rather than freshly fertilised eggs, which might have led to a starvation distress at the end of testing period (9d post-hatch in total rather than the suggested 5d post-hatch according to OECD TG 212), due to the absence of external feeding. Additionally, there was a lack of bodyweight measurements.

The DS recognised that the OECD TG 212 recommends initiating the test as early as possible after fertilisation but also noted that the test protocol considers that no feeding is necessary and that no starvation is expected as long as the yolk sac nourishes the larvae. The DS concluded that these conditions were unlikely causing an overestimation of the observed effects, as revealed by 100% survival in the control group. Additionally, according to the OECD TG 212, a delay in the testing might influence the sensitivity of the test, however the suggested test duration ranges from the early gastrula stage to 5d post-hatch, corresponding to an interval of 8 - 10 days in total.

In this respect, RAC notes that the OECD TG 212 does not require the external feeding of the embryos, which is not expected to influence the outcome as long as the yolk sac is present. RAC recognises that the OECD TG 212 suggests initiating the testing as soon as possible after eggs fertilization (from 30 min to 8h), however RAC also notes that the yolk

sac provides sufficient nourishment for approximately 10 days after fertilization (according to the OECD TG 212 indications, 13 days at 25°C). Even assuming that 48 hpf larvae were used in the study CA 8.2.2.1/002 (2000), at the end of the testing period (7d) this would lead to an overall 10d post fertilisation, which is still within the acceptable range of 10 - 13 days. Taking into account that the survival of the control group was 100% at the end of the testing period, RAC deems unlikely that the absence of embryos feeding at the end of testing period might have influenced the biological outcome to a significant extent.

- **lack of information on post-hatch survival**: according to this comment, a comparison with the respective OECD TG 212 provision cannot be performed.

RAC notes that the study was conducted on already hatched larvae, rather than on early-fertilised eggs and, for this reason, the authors did not provide information on the post-hatch survival rate of the organisms in the control group. Nevertheless, since the survival rate of the larvae in the control group at the end of testing was 100%, RAC considers the lack of this information not relevant.

- overall **validity and reliability issues of the derived effects values**: according to this comment, the derivation of a NOEC for lethargy and mortality may not be relevant due to the poor description of the observed lethargy (in terms of individuals affected and their related alterations), and also questioning the statistical significance of the NOEC value. As an alternative to the NOEC of 1 mg/L, the use of EC_{10} was proposed.

RAC notes that further statistical analyses of the data from this study have been performed, deriving EC_{10} values for mortality. In this respect, RAC derived an EC_{10} performed using the Mosaic statistical package resulting in a value of 3.98 mg/L (95% CI: 2.27 - 6.13 mg/L). This value is consistent with the EC_{10} derived by the Applicant (3.31 mg/L, using a probit analysis) and one MSCA (4.6 mg/L, based on a 2-parameter log-normal model, R drc package, version: 3.0.1).

RAC considers that the derived EC_{10} based on mortality does not allow the NOEC value proposed by the DS to be discarded. In RAC's view, the preference of the NOEC over the EC_{10} for this specific case and environmental dataset is justified by the combination of two distinct endpoints, the first being mortality and the second lethargy, that concomitantly are observed after 7d exposure to 3.2 mg/L glyphosate concentrations.

RAC acknowledges that lethargy was poorly described in terms of morphological/behavioural alterations and number of affected individuals, not allowing the derivation of statistically robust EC_{10} or NOEC values. However, RAC notes that lethargy is an important alteration of behaviour that can potentially affect fish survival and reproductive capacity, thus representing a relevant chronic endpoint.

In the case of mortality, for which an EC_{10} can be derived, 10% of the tested embryos at 3.2 mg/L were dead while a certain, yet undefined, number of individuals became lethargic. The lack of quantitative information does not allow to derive an EC_{10} for the endpoint lethargy. Taking into account that these biologically relevant effects were relevant for zebrafish survival and reproduction, RAC proposes to set the LOEC value to 3.2 mg/L, despite the lack of statistical significance. Consequently, RAC considers that retaining a NOEC value of 1 mg/L based on mortality and lethargy combined, instead of the EC_{10} based on mortality alone and/or looking at the individual effects endpoints separately, would be the most scientifically robust assessment of all available information, in agreement with the principles of the CLP Regulation.

Table: *Comparison of experimental design and validity criteria between OECD TG 212 and study CA 8.2.2.1/002 (2000)*

	OECD TG 212	CA 8.2.2.1/002 (2000)
Validity criteria	Post-hatch survival of fertilised eggs in the control ≥ 90%	No info (larvae exposed during the test and not fertilised eggs, survival in the control 100%)
	Dissolved oxygen between 60% and 100% of air saturation	50 - 70% at 24°C
	Water temperature 25 ± 1°C (difference between test chambers and/or successive day < 1.5°C)	23.0 - 24.2°C
Specification	Less sensitive to chemicals with log K_{ow} >4 and chemicals with specific Mode of Action	
Start	Fertilised eggs (30 min post fertilisation)	Not clear which larvae stage was used (how long after fertilisation) No info on hatching success of eggs, nor on fish weight at the beginning and the end of testing period
Duration	For zebrafish 8 - 10 days	7 days
Test conditions	Static/semi-static/FT	Semi-static with renewal of test solution each 48h
Test solution	By dilution of a stock solution	By dilution of a stock solution
Eggs number	At least 30 eggs divided by at least 3 replicates per conc.	3 replicates with 10 larvae per replicate. 30 individuals in total per concentration
Tested concentration	5 concentrations	7 concentrations: 0, 0.32, 0.56, 1.0, 3.2, 5.6, 32 mg/L
Analytical determination	Determination of test substance concentrations prior to renewal need only be performed on one replicate vessel at each test concentration. When conc. remains within ± 20% of nominals - the highest and lowest test concentrations be analysed when freshly prepared and immediately prior to renewal on at least three occasions spaced evenly over the test (i.e. analyses should be made on a sample from the same solution - when freshly prepared and at renewal).	The active ingredient analysis of stock solution was determined by liquid chromatography, its concentration was at least 80% of nominal value throughout the test (on average 97%). Apparently, glyphosate concentration in solution was measured separately at stock concentrations of 100 and 1000 mg/L. Only water quality measurements available for one of test vessels.
Test parameters	During the test, dissolved oxygen, pH and temperature should be measured in all test vessels. Total hardness and salinity (if relevant) should be measured in the controls and one vessel at the highest concentration. In semi-static tests, it is recommended that dissolved oxygen be preferably before and after each water renewal or at least once a week. pH should be measured at the beginning and end of each water renewal in semi-static test and at least weekly in flow-through tests. Hardness should be measured once each test. Temperature should be measured daily and it should preferably be monitored continuously in at least one test vessel.	Test conditions monitored in all test vessels and maintained throughout the test: temperature, conductivity, pH, dissolved oxygen conc. Hardness apparently measured in a separate vessel up to 7d

	OECD TG 212	**CA 8.2.2.1/002 (2000)**
Observations for mortality	Observations on survival should be made at least once daily	Every 24h
GLP		yes
NOEC	Can be derived	1 mg/L based on mortality/lethargy (no raw data available for lethargy) according to the DS. 3.2 mg/L based on mortality (raw data available) according to the authors
LOEC	Can be derived	3.2 mg/L according to the DS 5.6 mg/L according to the authors

CA 8.2.2.1/001, 2010

Another comment regarded the chronic study conducted on Rainbow trout (CA 8.2.2.1/001, 2010) that was considered valid for the use in risk assessment, in the parallel ongoing processes under the PPP Regulation. It was suggested to consider the NOEC of 9.6 mg/L as relevant for the purpose of glyphosate classification, disregarding the study CA 8.2.2.1/002 (2000) due to the presence of uncertainties.

As explained above, RAC considers that the study CA 8.2.2.1/002 (2000), despite some uncertainties and minor deviations, is sufficiently robust and reliable and can be used as key information for the purpose of classification. RAC notes that even though the study CA 8.2.2.1/001 (2010) is valid, it is affected by some shortcomings that make it not sufficiently reliable, as already discussed in the Dossier Submitter proposal section. Therefore, in agreement with the principles of the CLP Regulation the lowest chronic endpoint is preferred in order to adequately protect aquatic organisms.

Fiorino et al. (2018)

This study was conducted according to a modified OECD TG 236 and assessed both lethal and sub-lethal effects of pure glyphosate in *Danio rerio* (zebrafish) and *Cyprinus carpio* (common carp) from 24 hpf up to 120 hpf. The data showed a significant alteration on the hatching rate of *Cyprinus carpio* embryos, at 0.05 mg/L, from 72 hpf onward and a significant increase in the cumulative mortality that peaked at 120h, wherein a significant difference with the control was seen at concentrations as low as 0.005 mg/L (lethal endpoints). Comparatively, statistically significant cumulative mortality in the zebrafish embryos was recorded already after 48h of treatment, yet at slightly higher concentrations of 0.05 mg/L. It should also be noted that a significant number of the treated carp embryos (58.3%) exhibited signs of delayed development after 120hpf of exposure to concentrations of glyphosate as low as 0.005 mg/L, which is a sublethal adverse effect of biological relevance. In comparison, at the same experimental time point, only 2.7% of the zebrafish embryos exhibited signs of late development in response to glyphosate concentrations of 0.05 mg/L, indicating a greater sensitivity of *Cyprinus carpio* under these experimental conditions.

The authors observed differences in the rate of cumulative mortality and malformations induced by acute glyphosate exposure in these two species which might reflect specific differences in the duration of their respective developmental stages, with zebrafish requiring 3 - 5 days to reach the sac-fry stage and carps comparatively needing longer time to reach independent feeding stage.

In RAC's view, the study provides relevant information on glyphosate toxicity despite the presence of some limitations. At first, the testing period of 120h covers a limited range of the

fish life-cycle and thus extrapolation of a NOEC value cannot be considered feasible. Also, the lack of experimental details on the overall fertilization rate and the dissolved oxygen concentration precludes a thorough comparison with the OECD TG 236 validity criteria. Additionally, the hatching rate in the control of both fish species was < 80%. Furthermore, the absence of raw data does not allow to derive a robust LC_{50} value at 96h that might be used for regulatory purposes, which hampers the use of the study for the purpose of aquatic acute classification.

RAC considered as not valid the results from *D. rerio* as the hatching rate in the control group (50%) was markedly below the threshold of the validity criteria (≥ 80%). In RAC's opinion, however, the study cannot be dismissed due to the occurrence of severe biological effects at very low glyphosate concentrations, as evidenced by the significant increase in the cumulative mortality of zebrafish and common carp embryos, after 96h of exposure to 0.05 mg/L and 0.005 mg/L, respectively. Moreover, in carp embryos, a significant alteration of the hatching rate was seen at 0.005 mg/L from 72h onward, while a significant proportion of the exposed embryos (58.3%) showed signs of delayed development after 120h of treatment, with a clear-dose response (100% of embryos at 5 mg/L). Thus, based on results on *C. carpio*, RAC considers the Fiorino *et al*. (2018) study reliable with restrictions and concludes that the biological effects observed on fish are relevant and can be used as supportive to the current classification proposal as Aquatic Chronic 2, even if quantifiable LC_{50} or NOEC values cannot be derived.

Zhang et al., 2021

In this study the effects of glyphosate and its primary transformation product AMPA on zebrafish embryos were tested. The embryos were exposed to 10, 100, or 700 ng/mL of glyphosate and AMPA from 2 to 72hpf. Concentrations as low as 10 ng/mL (0.01 mg/L) of glyphosate or AMPA decreased the hatch rate and survival of the embryos (lethal endpoints) at 72hpf. The authors also observed an increased rate of developmental malformations (sub-lethal endpoint) after 72h of treatment, deriving a LOEC of 10 ng/mL (0.01 mg/L).

RAC notes that the study can be compared to the OECD TG 236 acute toxicity test, yet with a shorter exposure duration of 72h instead of the usual 96h. RAC also notes that the study lacked detailed information on parameters useful for a direct comparison with the validity criteria, including the overall fertilization rate of the eggs and the concentration of the dissolved oxygen. The water temperature slightly exceeded (28 ± 0.5°C) the suggested value (26 ± 1°C) and no positive control was used. By contrast, the overall survival rate and the hatching rate in the negative control were in the suggested range (although they were measured at 72h instead of 96h).

Due to these limitations, RAC notes that the study from Zhang *et al*. (2021) does not allow to derive a LC_{50} to be used for the acute classification of glyphosate nor a NOEC value for the purpose of chronic classification. However, RAC considers the study relevant since a significant decrease in the survival and hatching rates was observed at glyphosate concentrations as low as 0.01 mg/L, that were paralleled also by a significant increase in the rate of malformations. Importantly, all these effects showed a clear dose-response although the interval between the exposure concentrations was not properly spaced. RAC considers that the severe effects observed at 72h and at very low glyphosate concentrations (0.01 mg/L) are indicative of chronic effects at similar or even lower doses and concludes that the study can be supportive to the current classification proposal of glyphosate as Aquatic Chronic 2.

Regarding the studies from Fiorino *et al.* (2018) and Zhang *et al.* (2021), their reliability and validity for the purpose of classification were questioned due to limitations in the experimental design (i.e. small test media volumes, lack of measurement for a number of physicochemical properties, deviations from OECD TG 236). RAC recognises that both studies are affected by these limitations in the experimental design and do not provide all the necessary information (i.e.

raw data and extensive measurements of all the required parameters) to derive quantitative EC_{50} or NOEC values to be used in the classification of glyphosate. Thus, in RAC's opinion and for the reasons explained above, they cannot be used as basis for acute glyphosate classification. However, RAC also considers that these studies can be used as a part of the overall evidence to support the chronic classification proposal of glyphosate since severe lethal and sub-lethal effects were observed at very low concentrations of the substance (in the order of 0.01 and 0.005 mg/L) already after 72 - 120h and chronic effects are expected to occur at similar if not lower exposure levels.

<u>Fan et al., 2022</u>

A species sensitivity distribution (SSD) approach proposed in Fan *et al.*, 2022 was also considered. The toxicity studies used to derive the SSD were assessed by RAC for reliability with ToxRTool evaluation standards. An acute HC_5 (Hazardous Concentration) of 8.13 mg/L was calculated over 42 species, chronic data from 19 different species provide a HC_5 of 0.55 mg/L. RAC notes some issues which make analysis and interpretation difficult, such as:

- the database collected studies on both glyphosate and glyphosate-based formulation,
- acute values were used for the chronic evaluation,
- biochemical markers were included as endpoints,
- for some studies the minimum test duration for chronic data is shorter than acceptable,
- the lack of raw data and therefore impossibility to verify test guideline validity criteria,
- poor description of test conditions,
- absence of "conventional" toxicological effect parameters.

In conclusion, RAC considers that the Fan et al. (2022) SSD HC_5 value is not suitable for the purpose of classification.

Aquatic invertebrate studies

Two studies assessing the chronic effects of glyphosate on the harlequin fly *Chironomus riparius* were not part of the original CLH dossier but were brought to the attention of RAC in the later stages of the process.

Study ECT-2019-0362 (2019) assessed the chronic (28d) effects of glyphosate on *C. riparius* in a water-sediment system with water-spiked administration. Following a non-GLP range finding test at 0.1 - 1000 mg/L, a definitive extended limit test was conducted at nominal concentrations of 100 or 1000 mg/L in compliance with GLP and based on OECD TG 219. A NOEC of 1000 mg/L (nominal concentration) was reported for both emergence ratio and development time. Not all OECD TG 219 validity criteria were fulfilled, with the main deviation consisting in a prolonged time required for midge emergence in controls ($\leq$ 28 instead of $\leq$ 23 days). Data on analytical verification from the definitive test are missing. Analytical control was performed only for overlying water of the range finding test, showing a decrease of up to 54.5% of nominal values, however analytical measurements in pore water and sediments were not performed. Therefore, the reported NOEC value of 1000 mg/L based on nominal concentrations cannot be considered by RAC as sufficiently reliable and relevant for classification purpose.

Study ECT-2020-0027 (2020), performed in compliance with GLP, assessed the chronic (28d) effects of glyphosate on *C. riparius* under sediment-spiked administration at concentrations between 1 and 640 mg/kg. The analytical verification of nominal concentrations was performed. NOEC values of 740 and 154 mg/kg (based on measured concentrations) were obtained for emergence ratio and development rate, respectively. All the validity criteria according to OECD TG 218 were fulfilled. However, ecotoxicity data expressed in mass of test item / mass of sediment are not suitable to derive effect endpoints in the framework of the CLH process. No information was provided on the effects due to the presence of glyphosate in overlying water and

pore water. For this reason, the study is not considered by RAC relevant for classification purposes.

Algae studies

A relevant algae study was not part of the original CLH dossier but was brought to the attention of RAC in the later stages of the process. Glyphosate effects were assessed in Monsanto unpublished study BN-78–44C (EG & G Bionomics, 1978) on the marine alga *Skeletonema costatum* and a 7d NOEC=0.534 mg/L for growth was reported. After the assessment of the full study report, RAC notes that the results do not contain the NOEC value reported in the JRC draft dossier (0.534 mg/L). In the provided study, toxicity effects of 7 different compounds (glyphosate plus other not-specified substances – no purity reported) were assessed. The study was poorly described, raw data were not available, the only effects assessed were chlorophyll *a* and cell number in percentages and it was not possible to verify the OECD TG 201 validity criteria. Furthermore, no analytical information was provided and there was no clear indication of test the guideline followed ("Culture and procedures followed those of U.S. Environmental Protection Agency (1976)"). The acute endpoint was a 96h EC_{50} of 1.3 mg/L for decrease in cell number (95% CI: 0.7 - 2.5 ppm). No chronic values $NOEC/EC_{10}$ were derived, but a decrease in both cell number concentration and chlorophyll *a* was 12% at the test glyphosate concentration of 0.6 mg/L was observed. Although this value is below 1 mg/L, the reliability of the study cannot be assessed. RAC therefore considers this study not adequate to derive reliable toxicity values for classification purposes.

Aquatic plant studies

Table: *Summary of the chronic values of the studies on aquatic plants outside the CLH dossier*

Method	Species	Test material	Results	Reference
ASTM E1913-97	*Myriophyllum sibiricum*	Glyphosate technical, 97%	Growth (shoot length) 14d NOE_rC: 0.332 mg/L (nom)	Roshon, 1997
OECD TG 239 GLP	*Myriophyllum spicatum*	Glyphosate technical, 96.0%	Wet weight 14d NOE_rC: 4.69 mg/L (mm) 14d E_rC_{10} of 5.41 mg/L (mm)	Study no. ECT 21P1MW, 2022
Maltby *et al*., 2008 (at the base of OECD TGs 238, 239)	*Myriophyllum aquaticum*	MON 52276 (glyphosate formulation, 30.7%)	Shoot fresh weight 14d NOE_rC < 0.3 mg a.e./L (mm) 14d E_rC_{10}=0.16 mg a.e./L (mm) Shoot length 14d NOE_rC=1.1 mg a.e./L (mm) 14d E_rC_{10}=1.07 mg a.e./L (mm) Shoot dry weight 14d E_rC_{10}=0.44 mg a.e./L (mm)	CP 10.2.1/006, 2012
OECD TG 221 (2006)	*Lemna gibba*	Glyphosate technical, 97.7%	Frond numbers 7d E_rC_{10}=3.79 mg/L (nom) 7d NOE_rC=3.05 mg/L (nom)	Study no. S21-00368, 2021

a.e.=acid equivalent

Roshon, 1997

The study by Roshon (1997) was a PhD thesis describing the toxic effects of several herbicides on the aquatic macrophyte *Myriophyllum sibiricum*. Concerning glyphosate, the ASTM E1913-97 guideline was followed, which, although not currently used, was the basis of the adopted OECD TG 238 and 239. Apical shoots 3 cm long, obtained from sterile plants of *M. sibiricum*, were potted into test tubes containing 3 g of the artificial sediment Turface® and 40 mL of nutrient medium previously spiked with the test item. Concentrate stock solution in water of glyphosate (97%) were added to the modified Andrews' medium to obtain the dilution series of 4.1, 12.3, 36.9, 110.6, 331.9, 995.6 or 2987 µg/L. Five replicates for each concentration were prepared compared to 4 control plants. In addition, a separate experiment was conducted using only the

highest test concentration of 2.99 mg/L compared to control plants. Plants were maintained under controlled environmental conditions for 14d during which time the increase in shoot length was measured and growth curves established. The other measured parameters were area under the growth curve, root length, root number, fresh weight, membrane integrity, plant area, chlorophyll *a* and *β* (apical dry weight) and carotenoid (apical dry weight). The statistical analysis was conducted based on nominal concentrations.

Even if there was a claim that this study resembled OECD TG 239 (i.e. based on a water-sediment system), instead RAC considers that this study was more similar to OECD TG 238, based on a sediment-free test system. Thus, comments comparing this study with OECD TG 239 (rather than OECD TG 238), are considered by RAC as not relevant. Thus, the use of a medium containing sucrose (Modified Andrews' medium) is considered within the specifications of OECD TG 238. Furthermore, the addition of an artificial clay substrate with the sole purpose for rooting, and not containing nutrients, can be supported by RAC.

It was also pointed out that the test species was not the standard test species and that no analytical control was performed. Regarding the test species, RAC notes that the test protocol ASTM E1913-97 was explicitly developed for this specific *Myriophyllum* species and considers both *M. sibiricum* and *Myriophyllum spicatum* as suitable species. Regarding the lack of analytical control, in RAC's opinion, glyphosate is proven to be sufficiently stable in water, therefore it is not expected to decrease during the 14d test period.

Within the study, two separate experiments were performed with the same test material: a seven-concentration dose response and a limit test with a single concentration. It was argued that results from the single concentration test, shown in table 64 of the publication, could not be used to verify the validity criteria of the seven-concentration dose response test. RAC recognises the lack of raw data for both experiments that does not easily facilitate the verification of the validity of the study. However, for the single concentration test, table 64 reported the mean values for the different endpoint parameters for both the test substance and the control, showing that the OECD TG 238 validity criteria are met: the mean total shoot length in control plants doubled before the end of the exposure period (shoot length was 2.9-fold at the end of the test) and the mean coefficient of variation for total fresh weight in control plants did not exceed 35% (CV was 4%). Regarding the seven-concentration dose response test, from which the NOEC is derived, visual observation of the growth curve for the increase in shoot length reveals clearly that results of control groups are comparable to that of the single concentration test, as well as results from the highest test concentration treated groups. For these reasons, the validity criteria are also considered by RAC as fulfilled for the experiment testing the seven concentrations.

It was also noted by commenting parties that measured parameters were based on yield and not on growth rate. However, RAC assessed the growth curve graph that allowed the derivation of a NOEC value also based on growth rate, in addition to the one based on yield.

The growth endpoints were based on percent reduction in yield and the lowest acute value was an EC_{50} of 0.844 mg/L for root length. In RAC opinion, this value cannot be used for the purpose of acute classification, as the effects on roots are considered only an additional determination to the key effect parameter of the OECD TG 238 which is main shoot length. Moreover, this guideline alerts against the use of root endpoints for chemicals having auxin-type mode of action, as exposure of roots to light during the test may have an influence on auxin transport carriers.

Using statistical analysis ($α=0.05$) a NOEC value of 0.332 mg/L was determined in the Roshon study for the area under the growth curve, increase in shoot length, root length, chlorophyll *a* and carotenoid content. The data showed a clear dose-response relationship for growth rate and, although the raw data were missing, based on the published graphics the changes in the logarithms of the mean shoot length divided by the test duration can be calculated. Thus, the NOE_rC value of 0.332 mg/L for increase in shoot length has been derived by RAC. Despite the

shortcomings of the study, RAC has concluded that the study is sufficiently robust and reliable and can be used as key information to confirm the proposed classification.

CP 10.2.1/006, 2012

In the RAR, several studies testing the glyphosate formulation MON-52276 (Vol. 1 table 2.9.2.2.5-1) are available. The formulation study providing the most conservative outcome was study CP 10.2.1/006 (2012) that was conducted on *Myriophyllum aquaticum*, in compliance with GLP. The study followed a draft guidance of SETAC AMRAP (the basis of OECD TGs 238, 239), the test item was formulation MON-52276 containing glyphosate 30.7% w/w and nominal concentrations ranged from 0.24 to 750 mg glyphosate/L; aquatic plants were exposed for 14d in a water-sediment system. All validity criteria were met. Acute and chronic endpoints were expressed in mean measured concentrations and were based on both yield and growth rate. The lower values were observed for shoot fresh weight 14d E_rC_{10}=0.16 mg a.e./L (mm) and for shoot dry weight 14d E_rC_{10}=0.44 mg a.e./L (mm).

One commentator supported the use of this study for the classification of glyphosate. The DS agreed with this observation but considered that even if the toxicities of the constituents in the formulation were known, it could not be determined precisely if the effects were mainly due to the active substance without a comparison with a study conducted with active substance alone.

RAC previously agreed the environmental classification of mecoprop-P (ECHA, 2019) based on a study on *Myriophyllum spicatum* with a formulation product. The reasons for that decision on that specific case were the specific mode of action of the active substance as an herbicide, the low concentration of the co-formulants, the similar toxicities of the active substance and the formulation product in other species. Regarding formulation MON-52276, five studies show that similar toxicities between the active substance and the formulated product can be observed for fish, aquatic invertebrates and aquatic plants (*Lemna* and *Myriophyllum*), although the information on the co-formulants was not provided. The lowest toxicity values were obtained on the aquatic plant *Myriophyllum aquaticum* (CP 10.2.1/006, 2012) where the chronic endpoints for shoot length, shoot fresh weight and dry weight were below 1 mg/L, these values are in the range of those obtained in Roshon (1997). Therefore, based on these studies, it is reasonable to state that the effect of glyphosate in that study is not influenced by the formulation MON-52276 and the results from the study CP 10.2.1/006 (2012) on *Myriophyllum aquaticum* can be considered as supportive evidence for the purpose of the classification.

RAC therefore considers the study reliable and proposes that the results can be used as evidence supportive of the current classification proposal as Aquatic Chronic 2.

ECT 21P1MW, 2022

Study no. ECT 21P1MW (2022) on *Myriophyllum spicatum* was conducted according to OECD TG 239 and is GLP compliant. The study was provided to ECHA after the end of the targeted consultation. Glyphosate effects on the growth of *Myriophyllum spicatum* were assessed in a 14-days sediment-water system. The test item was applied into the water phase at 1.58, 5.00, 15.8, 50.0, 158 or 500 mg/L. The lowest NOEC value among the different measured parameters was 4.96 mg/L for total shoot length (yield) and biomass wet weight (yield and growth rate), the lowest EC_{10} values were 3.00 and 5.41 mg/L for yield and growth rate biomass wet weight, respectively; the results were based on geometric mean measured concentrations. All the validity criteria for OECD TG 239 guidelines were fulfilled and RAC considers the study reliable, and it was considered together with all other reliable studies when concluding on chronic classification.

A table comparing the three *Myriophyllum* studies reported above with the OECD TGs 238 and 239 guidelines is presented below.

Table: *Comparison of experimental design and validity criteria between the three studies on Myriophyllum species not included in the CLR report and the OECD TGs 238 (sediment-free Myriophyllum spicatum toxicity test) and 239 (water-sediment Myriophyllum spicatum toxicity test)*

OECD TGs 238 and 239	Roshon, 1997	CP 10.2.1/006, 2012	Study no. ECT 21P1MW, 2022
Species *Myriophyllum spicatum*	*Myriophyllum sibiricum*	*Myriophyllum aquaticum*	*Myriophyllum spicatum*
Test item	Technical glyphosate, 97%, batch information not available	MON52276 Glyphosate formulation, 30.7% , batch information available	Technical glyphosate, 96%, batch information available
GLP	No	Yes	Yes
Test guideline	ASTM E1913-97 (withdrawn, but at the base of OECD TGs 238, 239)	Maltby *et al.*, 2008: Aquatic Macrophyte Risk Assessment for Pesticides, SETAC AMRAP	OECD TG 239 (2014)
Duration of exposure	14d	14d	14d
Substrate (only for OECD TG 239) Artificial sediment described in OECD TG 219 with addition of nutrients	Turface® (clay substrate used in OECD TG 238 for the maintenance of stock culture)	Compliant with OECD TG 239	Compliant with OECD TG 239
Medium Modified Andrews' medium (OECD TG 238) Smart and Barko medium (OECD TG 239)	Modified Andrews' medium	Smart & Barko medium	Smart & Barko medium
Considered effects shoot length, fresh weight and dry weight, as well as qualitative observations of symptoms such as chlorosis, necrosis or growth deformities in addition, only for OECD TG 238: lateral branches and roots, increase of whorls	Shoot height, root number, length, growth curve, primary productivity, fresh weight, dry weight, plant area, chlorophyll (a, β), carotenoid, membrane integrity, as well as visual effects	Shoot length, fresh weight and dry weight, root length	Shoot length, fresh weight and dry weight, qualitative observations of symptoms such as chlorosis, necrosis or growth deformities
Tested concentrations minimum of five tested levels, spacing factor not exceeding 3.2	4.1, 12.3, 36.9, 110.6, 331.9, 995.6, 2987 µg/L (S.F.=3)	0.24, 1.2, 6, 30, 150, 750 mg glyphosate/L (S.F.=5) nom0.3, 1.10, 5.16, 26.8, 145, 723 mg glyphosate/L mm	1.58, 5, 15.8, 50, 158, 500 mg glyphosate/L (S.F. < 3.2) nom1.30, 4.96, 12.7, 39.6, 132, 445 mg glyphosate/L mm
Analytical verification at least at test initiation and termination in both water and sediment – in sediment at least for the highest treatment	No ("The statistical analysis was conducted based on the nominal glyphosate concentrations since glyphosate residues usually do not decline in sterile water")	At test initiation and termination for all the tested levels	At test initiation and termination for all the tested levels
Validity criteria doubling of mean total shoot length and fresh weight, no symptoms of chlorosis, no contamination from other organisms (for OECD TG 238, > 50% plants are kept sterile), mean coefficient of variation for fresh weight yield in the control < 35%	Yes (shoot length 2.9-fold, coefficient of variation for ASTM E1913-97 were met (> 60% surviving replicates of the controls and treatments)	Yes (shoot length 2.6-fold, shoot fresh weight 2.62-fold, no symptoms of chlorosis, no contamination, coefficient of variation=16.5%)	Yes (shoot length 5.2-fold, shoot fresh weight 4-fold, no symptoms of chlorosis, no contamination, coefficient of variation=13.5%)
N. individuals/replicates at least 5 replicates, 1 individual per replicate (OECD TG 238)	5 replicates in tested levels and 4 replicates in the control, 1 shoot per replicate	5 individuals per replicate, 6 replicates for control and 3 replicates for tested levels	6 replicates in the control and 4 in tested levels, 3 shoots per replicate

OECD TGs 238 and 239	Roshon, 1997	CP 10.2.1/006, 2012	Study no. ECT 21P1MW, 2022
at least 6 replicates in the control and 4 in tested levels, 3 shoots per replicate (OECD TG 239)			
pH In a range of 6-9 (OECD TG 238) between 7.5 and 8 at test initiation (OECD TG 239)	Adjusted to 5.8 ± 0.1	Adjusted to 7.5 to 8.0 at test initiation	7.9 at test initiation
Light irradiance 100-150 $\mu E \cdot m^{-2} s^{-1}$, photoperiod 16:8 (OECD TG 238) 140±20 $\mu E \cdot m^{-2} s^{-1}$, photoperiod 16:8 (OECD TG 239)	Photon fluence rate 100 - 150 $\mu mol \cdot m^{-2} s^{-1}$ photoperiod 16:8	7295 - 7518 lux, photoperiod 16:8	140±20 $\mu E \cdot m^{-2} s^{-1}$, photoperiod 16:8
Temperature 23 ± 2°C (OECD TG 238) 20 ± 2°C (OECD TG 239)	20 - 25°C	20°C	18.5 – 21.8°C
Results (most sensitive)	14d NOE_rC=331.8 µg glyphosate/L shoot length (nom)	14d E_rC_{10}=1.07 mg glyphosate/L (mm) shoot length 14d E_rC_{10}=0.16 mg glyphosate/L (mm) shoot fresh weight 14d E_rC_{10}=0.44 mg glyphosate/L (mm) shoot dry weight	14d E_rC_{10}=38.2 mg glyphosate/L (mm) shoot length 14d E_rC_{10}=5.41 mg glyphosate/L (mm) shoot fresh weight
Raw data	No (only a graph and a table with endpoints)	Raw data available in the report	Raw data available in the report
Statistical evaluation	Both parametric t-test and nonparametric Mann-Whitney U-test	Detailed statistical analysis reported	Detailed statistical analysis reported

S21-00368, 2021

Another study on the duckweed *Lemna gibba* was conducted following OECD TG 221 and was claimed as GLP compliant. Five concentrations of technical glyphosate (0.954, 3.05, 9.77, 31.3 or 100 mg/L) were assessed over 7d under static conditions. Results were calculated using the nominal concentrations, since analytical controls showed that measured concentrations were between 80 - 120% of nominal. The lower acute and chronic values were obtained for frond number: E_rC_{50}=28.7 mg/L; E_rC_{10}=3.79 mg/L and NOE_rC=3.05 mg/L. Validity criteria were fulfilled and RAC considers the study reliable.

Amphibians

One commentator pointed out that several amphibian studies tested with aquatic life stages were considered reliable or reliable with restrictions in the terrestrial vertebrates' section of the CLH dossier. This data included a NOEC for glyphosate of 0.0006 mg/L based on survival and the relevance of these data for classification should be considered (Williams *et al.*, 2010, CA 8.1.4 in CLH dossier). The DS agreed to consider toxicity data on the aquatic phase of amphibians for classification purposes, as the CLP Regulation states that data on other species can also be considered if the test methodology is suitable. Nevertheless, the DS did not consider the study in question suitable for aquatic classification due to several limitations (lack of analytical measurements, formulation study, etc.). Two formulations were tested and due to other constituents besides glyphosate (e.g. surfactants) present in the formulations the results of the study are not considered representative of glyphosate toxicity.

RAC agrees with the DS to not consider these studies on amphibians further.

Assessment and comparison with the classification criteria

Degradation

RAC agrees with the DS's view to consider glyphosate as not rapidly degradable.

- The substance is not readily biodegradable. Biodegradation in the OECD TG 301F test was 26% after 28 days which is well below the pass level of 60% of the test.

- The substance was not ultimately degraded in a surface water simulation test with the half-life of < 16 days corresponding to a degradation of > 70% within 28 days. In the OECD TG 309 test, DT_{50} values were 12.3 and 21.8 days for the low and high dose, respectively. Mineralisation, however, was 23.1 - 26.5% AR after 62 days.

- In the water/sediment studies the degradation half-lives for the total system were from 8.4 to 195.8 days, mineralisation after 100 days was from 5.9 to 48% AR.

- The substance was stable to hydrolysis.

Bioaccumulation

RAC agrees with the DS to consider glyphosate as having a low potential for bioaccumulation.

- There is no reliable BCF for fish available. The BCF test available shows, however, some indication of low bioaccumulation potential.

- The log K_{ow} values for glyphosate range from -6.86 to < -0.7. The values are below the cut-off value in the CLP Criteria of log $K_{ow} \geq 4$.

Toxicity

Glyphosate is not rapidly degradable and does not fulfil the criteria for bioaccumulation. Based on the available and reliable information in the CLH dossier on acute toxicity, studies for the three trophic levels fish, invertebrates and algae/aquatic plants, the $L(E)C_{50}$ values are all above the threshold of 1 mg/L of the CLP criteria. Therefore, RAC agrees with the DS that **no classification as Aquatic Acute is warranted for glyphosate**.

For the reasons explained in detail in the previous sections, RAC agrees with the DS conclusion that the NOEC value of 1 mg/L on fish from study CA 8.2.2.1/002, 2000 is still relevant. RAC also considers relevant and reliable the study on *Myriophyllum sibiricum* (Roshon, 1997), with the NOEC value of 0.332 mg/L also warranting an Aquatic Chronic 2 classification. This conclusion is supported by the results from the fish studies of Fiorino *et al.* (2018) and Zhang *et al.* (2021), as well the study on *Myriophyllum aquaticum* (CP 10.2.1/006, 2012) based on a formulate (MON 52276).

RAC agrees with the DS conclusion that glyphosate warrants **classification as Aquatic Chronic 2.**

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ANNEXES:

Annex 1   The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH dossier prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2   Comments received on the CLH dossier, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).

Annex 3   Records of the targeted consultation following the identification of additional documents potentially relevant to the classification of glyphosate