Australian Government
Australian Pesticides and
Veterinary Medicines Authority

2016

Review of IARC Monograph 112 (Glyphosate): Tier 2

This publication is based on the draft review prepared by the Office of Chemical Safety (Department of Health)

ISBN 978-1-925390-51-3 (electronic)

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EXECUTIVE SUMMARY

The World Health Organization's (WHO) International Agency for Research on Cancer (IARC) released Monograph 112: Some organopohosphate insecticides and herbicides: Diazinon, Glyphosate, Malathion, Parathion and Tetrachlorvinphos, which presents a summary of the IARC's conclusions relating to those chemicals on 20 March 2015. The full glyphosate Monograph (hereafter referred to as the monograph) was published on 29 July 2015.

IARC have classified glyphosate as a Category 2A 'probably carcinogenic to humans' agent. This classification was based on:

- limited evidence of carcinogenicity in humans
- sufficient evidence of carcinogenicity in animals
- strong evidence that glyphosate can operate through two key characteristics of human carcinogens and that these can be operative in humans—genotoxicity and oxidative stress.

The Australian Pesticides and Veterinary Medicines Authority (APVMA) requested the Office of Chemical Safety (OCS) undertake a preliminary scoping review of the monograph to ascertain the relevance of the carcinogenicity classification and any implications this may have to the approval of glyphosate and registration of glyphosate formulations in Australia (nominated as Tier 1). In particular, as part of this preliminary review, the OCS was requested to identify any relevant data not previously considered by the APVMA.

The recommendation made by OCS as part of the Tier 1 review identified 19 scientific journal articles relevant to the carcinogenicity classification of glyphosate that had not been reviewed by OCS. The OCS recommended that these 19 studies be reviewed in detail as part of a Tier 2 assessment of glyphosate.

A draft Renewal Assessment Report (RAR) on glyphosate was published by the European Food Safety Authority (EFSA) in 2013. The final RAR report, including an addendum that considered the IARC re-classification of glyphosate, was published in 2015 and considered 16 of the 19 studies identified by OCS as warranting further assessment. Where appropriate, the evaluation outcomes published in the final EFSA RAR (or addendum) were considered together with the OCS critical appraisal of the study to determine the regulatory value of the study.

Based on the critical appraisal and the limited studies evaluated during the Tier 2, Part 1 assessment, there appears to be no new information indicating that glyphosate poses a carcinogenic risk to humans, on the basis of the following:

- a carcinogenic mechanism of action via genotoxicity or oxidative stress has not be been clearly demonstrated
- the level of cytotoxicity associated with in vitro genotoxicity testing of glyphosate was significant, limiting the ability of in vitro tests to determine genotoxicity potential of glyphosate.

The OCS noted some evidence from in vitro studies that glyphosate-based formulations are more toxic and/or genotoxic to cells than glyphosate technical; however, there was no data appraised in this review to confirm this effect *in vivo*. Furthermore, many of the studies exhibited significant methodological deficiencies, limiting the usefulness of the data to elucidate the genotoxic potential of glyphosate-based formulation. The toxic effects of detergents (surfactants) on cells *in vitro* are well documented, are not unique to glyphosate products and may not be relevant *in vivo*. Surfactants are generally not considered suitable for inclusion in *in vitro* assays as they are

capable of disrupting cell membrane integrity and permeability, causing cytotoxicity and inducing apoptosis, which may confound analytical results.

There is limited information regarding the involvement of an oxidative stress mechanism for inducing cytotoxicity by glyphosate. Hence, no definitive conclusions could be drawn on the ability of glyphosate-based formulations and their associated impurities to induce oxidative stress.

The OCS did not critically appraise a number of epidemiology studies or studies that focussed specifically on the toxicological or carcinogenic effects of glyphosate-based formulations as recommended as part of the Tier 2, Part 2 assessment, as this data was critically evaluated in detail by the international Joint WHO/Food and Drug Administration of the United Nations (FAO) Meeting in Pesticide Residues (JMPR) review.

1.1 OCS recommendations to APVMA

Based on the scientific appraisal of 19 studies identified to be critical to the IARC's carcinogenicity classification of glyphosate, the OCS concluded that there is insufficient information to indicate that it poses a carcinogenic risk to humans.

2 PREVIOUS OCS EPIDEMIOLOGICAL REVIEW IN 2005

Case-control human studies, which are described below, have previously suggested a statistical association between self-reported glyphosate use and an increased risk of non-Hodgkin's lymphoma (NHL).

The first report of an association of glyphosate exposure with NHL was from a case-control study conducted in Sweden; however, the assessment was based on only four exposed cases and three controls (Hardell & Eriksson 1999). A pooled analysis of this initial study with a study of hairy cell leukaemia (a rare subtype of NHL) suggested a relationship between glyphosate exposure and an increased risk of the disease (unadjusted analysis with an odds ratio (OR) of 3 and a 95% confidence interval (CI) of 1.1–8.5) (Hardell et al. 2002). A more extensive study across a large region of Canada calculated an increased risk of NHL associated with glyphosate use of 2 days or more per year, based on 23 exposed cases and 31 controls (OR = 2.1; 95% CI 1.2–3.7) (McDuffie et al. 2001). In a pooled analysis of case-control studies conducted in the US, De Roos et al. (2003) reported an association between glyphosate exposure and increased NHL risk in men after adjustment for other commonly used pesticides, based on 36 exposed cases and 61 controls (OR = 2.1; 95% CI 1.2–4.0).

By contrast, in another cohort study, De Roos et al. (2005) reported that glyphosate exposure was not associated with increased NHL risk in men after adjustment for other commonly used pesticides, based on 92 exposed cases. This finding is not consistent with the studies described above. One plausible explanation is that all previous studies reported lower numbers of exposed cases and were retrospective in design, thereby rendering the study susceptible to recall bias of exposure reporting. As information on exposures is obtained by questionnaires and interview of farmers or their next-of-kin, often years after the event, the quality of data on pesticide use (ie exposure) obtained by recall is limited (Blair et al. 2002). Indeed, recall bias is particularly problematic for widely used products like Roundup® and the potential for recall bias and for misclassification of pesticides were acknowledged as limitations in all such studies. On the other hand, the study by De Roos et al. (2005) reported a higher number of exposed cases and was prospective in design, which should have largely eliminated the possibility of recall bias. On this basis and also based on the toxicity profile of glyphosate based on animal studies, it is unlikely that exposure to glyphosate is associated with an increased risk of NHL.

This is further supported by a recent epidemiological report showing that the incidence of NHL decreased between 1991-2000 in Sweden, Finland, Denmark and the United States of America (Hardell & Eriksson 2003), a period in which glyphosate use increased very significantly. It is of interest to note that decreased NHL incidence during this period in Sweden also coincided with a decline in the prevalence of human immunodeficiency virus (HIV), which has been shown to be a risk factor for NHL (Pluda et al. 1993).

No further epidemiology studies were reviewed by OCS in this Tier 2 assessment of glyphosate.

3 TIER 2 REVIEW OF THE IARC MONOGRAPH 112 (GLYPHOSATE)

3.1 Tier 2 work plan background

The studies included in this Tier 2 assessment were selected using the specific inclusion criteria outlined in the Tier 1 assessment report. The abstracts of the studies cited in the IARC Monograph reference list were screened to determine their relevance to the carcinogenicity classification of glyphosate. This resulted in the selection of 19 studies that referenced glyphosate, carcinogenicity, genotoxicity or oxidative stress in the article abstract (Table 6).

This Tier 2 assessment provides additional data to facilitate the weight-of-evidence assessment of the carcinogenicity of glyphosate and included the review of studies relevant to carcinogenicity classification of glyphosate:

- 3 studies not reviewed by OCS or internationally
- 16 studies that were reviewed in the EU (EFSA 2015) report.

Glyphosate active was the main focus of this review; however, due to the overlap between glyphosate and glyphosate-based formulations in the IARC Monograph 112 and published papers, some results for glyphosate-based formulations are presented in this report.

3.2 Integration of international assessment reports

Tier 2 assessment

Some of the studies identified as critical to the re-evaluation of the carcinogenicity of glyphosate have also been evaluated by EFSA as part of a RAR undertaken by the member state Germany in 2013 (EFSA 2015).

For those studies that have been evaluated by EFSA (2015), OCS reviewed the conclusions and commented on whether it agreed with the EFSA RAR (2015).

The EFSA RAR (2015) concluded that glyphosate did not pose a carcinogenic or genotoxic risk to humans.

3.3 Findings of the Tier 2 assessment

Carcinogenicity

Limited epidemiology studies were evaluated as part of the Tier 2 assessment as a consequence of the specific exclusion criteria outlined in the Tier 1 assessment, as they either did not directly reference glyphosate in the abstract or the abstract included a reference to a glyphosate-based formulation. As a result, no additional data regarding the carcinogenicity potential of glyphosate in humans was identified in this report.

There was no additional data identified during this review to indicate that glyphosate is carcinogenic in humans. The available animal data also did not indicate that glyphosate is carcinogenic potential in rats and mice.

Genotoxicity and oxidative stress

The overall conclusion from the scientific journal articles that held an acceptable level of regulatory value and investigated the genotoxicity of glyphosate was that glyphosate does not pose a genotoxic risk to humans.

Similarly, no reliable mechanistic or *in vivo* data were available to support the proposal that glyphosate is genotoxic via a mechanism involving oxidative stress within the scientific journal articles that held an acceptable level of regulatory value.

Both of these conclusions are further supported by the lack of evidence for carcinogenicity in animal bioassays.

Of the carcinogenicity and genotoxicity studies included in this assessment, six articles reported results that were inconsistent with the current OCS evaluation and EFSA RAR (2015) conclusions (Bolognesi et al. 1997; Alvarez-Moya et al. 2001; Monroy et al. 2005; Gasnier et al. 2009; Manas et al. 2009; Prasad et al. 2009). Five of these six papers were determined to have limited regulatory value and the remaining one was determined to have significant methodological limitations. Indeed, of the 19 papers reviewed, only three reported a positive response but due to the methodological limitations, the regulatory value of these papers was considered limited (Table 1: highlighted in grey).

Table 1: Summary of conclusions for glyphosate based on the critical appraisal of specified studies

Reference	Outcome
Carcinogenicity	
JMPR (2004)	'unlikely to pose a carcinogenic risk to humans'
Greim et al. (2015)**	Not carcinogenic based on lack of evidence seen in animal studies.
Genotoxicity	
Alvarez-Moya et al. (2014)	Not suitable for regulatory purposes
Bolognesi et al. (1997)	Not suitable for regulatory purposes
Chan & Mahler (1992)	No evidence of genotoxicity; however limited regulatory value due to reporting or methodological deficiencies.
Gasnier et al. (2009)	Not suitable for regulatory purposes
Kier & Kirkland (2013)	Limited evidence of genotoxic effect at levels not expected under 'normal' exposure conditions
Li & Long (1988)	No evidence of genotoxicity
Manas et al. (2009)	Not suitable for regulatory purposes
Mladinic et al. (2009a)	Limited evidence of genotoxicity (comet assay) and increased oxidative activity; however limited regulatory value due to reporting or methodological deficiencies.
Mladinic et al. (2009b)	Not suitable for regulatory purposes

Reference	Outcome
Monroy et al. (2005)	Limited evidence of genotoxicity in one cell line; however limited regulatory value due to reporting or methodological deficiencies.
Prasad et al. (2009)	Not suitable for regulatory purposes
Rank et al. (1993)	No evidence of genotoxicity in the mouse micronucleus test *
Oxidative stress	
Astiz et al. (2009)	Not suitable for regulatory purposes
Chaufan et al. (2014)	No evidence of oxidative stress carcinogenic mechanism of action; however limited regulatory value due to reporting or methodological deficiencies.
Elie-Caille et al. (2010)	Not suitable for regulatory purposes
Gehin et al. (2005)	Not suitable for regulatory purposes
Kwiatkowska et al. (2014)	No evidence of oxidative stress carcinogenic mechanism of action

*Other genotoxicity results (Ames and Chromosome Aberration assays) were not considered reliable for regulatory purposes

**Assessment presented in Appendix C

Other findings

Some of the studies evaluated included additional data on glyphosate-based formulations, metabolites or impurities.

Limited evidence of genotoxicity of a glyphosate-based formulation in mice (Prasad et al. 2009) was provided, but glyphosate technical was not specifically investigated. Similarly, Chaufan et al. (2014) supported the conclusion that a glyphosate-based formulation (but not glyphosate technical) appears to induce cellular oxidative stress leading to increased cytotoxicity.

Kwiatkowska et al. (2014) provide supportive evidence that glyphosate impurities (specifically N-(phosphonomethyl)iminodiacetic acid; PMIDA) are cytotoxic to human erythrocytes via an oxidative stress mechanism. Similarly, Gehin et al. (2005) provided a potential mechanism of glyphosate-based formulation cytotoxicity (ie via oxidative stress) in human erythrocytes, as the data indicated an increased oxidative response when cells are exposed *in vitro*. However, due to methodological limitations, the biological relevance of these results is unclear.

Kier & Kirkland (2013) reported that glyphosate-based formulations are more genotoxic than glyphosate technical, which seemed to be related to excipients rather than the glyphosate technical itself. Kier & Kirkland (2013) also concluded that the positive results reported in sister chromatid exchange (SCE) and comet assays were associated with higher levels of cytotoxicity.

4 ASSESSMENT OF IDENTIFIED STUDIES

A number of general scientific principles are important for interpreting toxicology studies to ensure that any observed effects are treatment-related and toxicologically-relevant (ie adverse). International guidance on interpreting toxicity has been published by the WHO (2009 & 2015) (see http://www.inchem.org/documents/ehc/ehc/ehc240_index.htm and http://www.who.int/foodsafety/publications/jmpr_guidance_document_1.pdf?ua=1). The criteria generally used to determine whether an effect is treatment-related and adverse are the presence of a dose- or concentration-response relationship, consistency of the effect, statistical significance, biological plausibility and natural variation and incidental findings.

The presence of a dose- (*in vivo*) or concentration-dependent (*in vitro*) response generally indicates that the observed effects are likely treatment-related. However, when assessing the carcinogenicity or genotoxicity of a substance, it is important that cytotoxicity is assessed concurrently to determine whether the treatment-related effects are indeed due to carcinogenicity (and are therefore true adverse findings) and not the effect of cell death, caused by cell toxicity mechanisms.

In a weight-of-evidence assessment, any observation should be reproducible and consistent: the strength of any finding will be increased if it can be demonstrated in more than one laboratory. Plausible patterns in the hierarchy of the results will also strengthen the finding; ie where a finding *in vitro* is reproduced *in vivo*.

Statistical analysis is a useful tool for detecting differences between groups exposed to a test compound or not. Biologically, this difference may be real or a chance or incidental finding. Hence, a statistically significant result on its own without an evaluation of its biological and ultimately toxicological relevance provides only limited insight into the possible effects of a chemical. In order to determine whether an effect is truly treatment-related and adverse, a number of other criteria must be met, as described above.

Consideration of historical control data is also an important aspect of assessing whether an effect is potentially treatment-related and abnormal. Historical control data is a compilation of the findings from age- and sex-matched control animals from a number of separate studies performed by the same laboratory and provides an indication of the normal or background frequency of tumours that occur in that species/strain of animals by chance.
A statistically significant increase in tumour frequency may be observed in treated animals when a lower than normal tumour frequency is observed in control animals in that study. Conversely, a non-significant result may be observed when a higher than normal tumour frequency is observed in the control group.

Human data is preferred to laboratory animal data because it generally enables the most accurate risk estimate for the target species. There are a range of human studies that may be available including volunteer studies, clinical trials, occupational exposure studies, poisoning case reports and epidemiological studies. When evaluating studies conducted using laboratory animals, those that use mammals are a more appropriate model for humans.

When evaluating the toxicological effects of pesticides, such as glyphosate, studies in which the chemical was administered via the oral, dermal or inhalational routes are the only relevant routes of exposure for humans. Subcutaneous (sc), intraperitoneal (ip) or intravenous (iv) administration are less relevant because these routes are not how humans are exposed when chemicals are used correctly (according to approved label directions).

All scientific studies and assessment reports are evaluated based on their scientific merits, which includes a consideration of the study design, quality of the results, interpretation and conclusions. However, studies that have been conducted according to principles of Good Laboratory Practice (GLP) and comply with international test guidelines (eg those produced by the Organisation for Economic Co-operation and Development; OECD) are preferred because of the assurance of their scientific quality.

4.1 Genotoxicity

Alvarez-Moya, C, Silva, MR, Ramirez, CV, Gallardo, DG, Sanchez, RL, Aguirre, AC & Velasco, AF 2014, 'Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms', Genet Mol Biol, vol. 37, pp. 105–10.

Alvarez-Moya et al. (2014) investigated the genotoxic potential of glyphosate in plant cells, fish erythrocytes and isolated human peripheral blood lymphocytes (PBLs) using the *in vitro* alkaline comet assay. The *in vitro* and *in vivo* studies using plant and fish cells were not considered relevant to mammalian toxicology and were not included in this evaluation. No international guidelines were cited. Alvarez-Moya et al. (2014) was considered by the EFSA RAR (2015), but a detailed critique was not provided.

Methodology

Human PBLs were extracted from blood samples taken from eight young students (age and sex not specified) who were not on current medical treatment and were not smokers, drug users or inhabitants of a previously contaminated area. Slides were prepared as per Singh et al. (1988) and exposed to 0, 0.7, 7, 70 or 700 µM glyphosate technical (equivalent to 0, 0.00012, 0.0012, 0.012 or 0.12 mg/mL) for 20 hours. The experiment was repeated twice.

The alkaline comet assay described by Singh et al. (1988) and Alvarez-Moya et al. (2001) was used to measure DNA strand breaks in PBLs. Nuclei and cells were observed at 10× magnification and tail length during migration was recorded and analysed using Comet assay software. Two slides containing 50 cells or nuclei per slide for each experimental point and control were evaluated (ie 100 cells or nuclei per experimental condition).

Results

Migration (tail length) of PBL DNA increased by 2.1- to 2.5-fold (compared with negative controls) with increasing concentrations of glyphosate (Table 2). The responses to different concentrations of glyphosate differed significantly ($p < 0.0001$) and were significantly different from the negative and positive controls ($p \leq 0.01$).

Table 2: DNA damage in isolated human peripheral blood lymphocytes following exposure to glyphosate (n=8) (from Alvarez-Moya *et al.*, 2014).

Glyphosate concentration (mg/mL)	Mean tail length ± standard deviation (µm)
0 (negative control)	20.38 ± 4.47
0.00012	42.86 ± 8.62
0.0012	42.27 ± 8.62

Glyphosate concentration (mg/mL)	Mean tail length ± standard deviation (µm)
0.012	48.93 ± 9.16
0.12	51.01 ± 10.43
0.51 NDEA (positive control)	44.26 ± 5.19

NDEA: N-nitrosodiethylamine

Discussion and OCS conclusion

The comet assay is commonly used to detect genetic damage in individual cells (Singh et al. 1988). The relevant guideline for use of the comet assay to assess DNA damage potential (OECD Test Guideline (TG) 489, 2014, released after the study was accepted for publication) involves *in vivo* exposure to the test substance followed by cell or nuclei isolation and subsequent analysis via the comet assay. In contrast, the study by Alvarez-Moya et al. (2014) exposed isolated human PBLs directly to the test substance and does not take into account *in vivo* absorption, distribution, metabolism and excretion (ADME). The use of isolated PBLs and direct exposure to assess genotoxicity is described in OECD TG 487 (*in vitro* mammalian micronucleus test); however, this TG describes the use of cells in suspension not in agarose on a prepared slide.

The EFSA RAR noted some reporting deficiencies (ie no indication of pH or osmolality was provided and results were not reported separately for replicate cultures) and that the results of the comet assay were inconsistent and did not demonstrate a clear concentration-dependent relationship.

The OCS noted a number of limitations in Alvarez-Moya et al. (2014) but also noted that it was not possible in some circumstances to determine whether these were reporting deficiencies or study design and conduct deficiencies. No validation studies were provided to demonstrate that the test system used to detect DNA damage following chemical exposure were suitably sensitive or that appropriate levels of cytotoxicity were maintained with the test conditions and cell/slide storage. No justification for the doses selected was provided (although the OCS noted that they covered a large range) and a number of highly relevant conditions were not described by the authors (vehicle used during test substance exposure, period of storage between various steps in the study protocol, treatment of cytotoxic artefacts, volume of cell suspension added to agarose, cell density on slides, levels of cytotoxicity). The data for 50 out of 100 cells recorded was selected for reporting purposes only and the reason for this was not provided. Furthermore, the age (or at least age range) and sex of the PBL donors was not provided, which may affect the interpretation of the results, as evidenced by the presence of micronuclei in isolated PBLs: DNA damage increases with age and is more marked in females than in males (OECD TG 487, 2014). The positive control used (N-Nitrosodiethylamine; NDEA; cited as per Alvarez-Moya et al. (2014)) was originally tested on *Tradescantia* (plant) cells and is not considered predictive for human genotoxicity, and exposure was prior to preparation of the cells for the comet assay.

A significant limitation of this study is the authors' extrapolation of the analysis of variance (ANOVA) results to imply that a dose-response relationship was demonstrated. The ANOVA statistical test identifies when one or more condition differs from the others and does not indicate trends. It does not identify which dose responses differ and does not take into account the order of the doses. The authors reported a significant increase in tail length (and hence DNA damage) for PBLs exposed to glyphosate at doses of 0.00012 mg/mL to 0.12 mg/mL compared with the negative and positive controls. However, it is difficult to compare these results with other studies as these concentrations were derived from direct exposure of PBLs and not *in vivo* exposure. The cells are

likely to be more sensitive to direct exposure and this *in vitro* cell culture model may not be biologically relevant to realistic exposure scenarios.

Finally, the OCS concluded that, given the absence of information that has a bearing on the level of cell death and a lack of reporting on the levels of cytotoxicity, conclusions relating to the genotoxic effect of glyphosate could not be determined.

Bolognesi, C, Bonatti, S, Degan, P, Gallerani, E, Peluso, M, Rabboni, R, Roggieri, P & Abbondandolo, A 1997, 'Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup®', Journal of Agricultural and Food Chemistry, vol. 45, pp. 1957–62.

Bolognesi et al. (1997) investigated the genotoxic effects of analytical grade glyphosate (300 mg/kg) and a glyphosate-based formulated product (Roundup®; 30.4% glyphosate) in both *in vivo* and *in vitro* test systems including:

- SCE in human PBLs
- DNA damage in Swiss CD-1 male mice (n=3 per dose) following administration with either glyphosate technical or 900 mg/kg Roundup® (approximately 270 mg/kg glyphosate) via ip injection
- micronucleus assays performed on two groups of three male Swiss CD-1 mice using glyphosate technical or 450 mg/kg Roundup® (approximately 135 mg/kg glyphosate) via ip injection twice, with a 24 hour interval period.

Glyphosate technical and Roundup® induced a significant increase in DNA single-strand breaks and alkali labile sites (approximately 3-fold compared with controls) in mice at 4 hours post treatment, which resolved to control levels at 24 hours post treatment. However, these results were presented graphically, without numerical data so it is difficult to determine the exact magnitude of the response. Glyphosate technical stimulated significant DNA oxidative metabolism in the liver at 24 hours post treatment as measured by a 6-fold increase in 8-OHdG (8-hydroxy-2'-deoxyguanosine), but did not stimulate a response in the kidney. In contrast, Roundup® stimulated significant DNA oxidation in the kidney at 8 and 24 hours post treatment as measured by a 2.3 and 3-fold increase in 8-OhdG, respectively, but did not induce a response in the liver. Again, these results were only presented graphically.

Glyphosate technical and Roundup® induced concentration-dependent increases in SCEs in human lymphocytes (1.9- and 1.5-fold, respectively, compared with controls interpreted from graphical results). The increase in SCE frequency observed at the highest concentration of Roundup® tested (0.33 mg/mL) was comparable with a glyphosate technical concentration at a ten-fold higher concentration (3 mg/mL).

Glyphosate technical and Roundup® induced a small but significant increase in bone marrow micronucleus frequency (3.2- and 4.1-fold, respectively, compared with controls) in mice.

The authors concluded that glyphosate technical and Roundup® have genotoxic potential and cause oxidative damage to DNA.

Discussion and OCS conclusion

The EFSA RAR (2015) noted that the micronucleus assay results reported by Bolognesi et al. (1997) are not consistent with other tests which have used higher doses and did not result in a genotoxic effects. EFSA further noted a number of critical reporting and/or methodological deficiencies. Only data without metabolic activation was generated in *in vitro* assays and no positive controls were included in *in vitro* SCE assay or *in vivo* experiments. The micronucleus assay was not performed according to contemporary guidelines (OECD TG 487) (eg only one concentration of glyphosate was tested, rather than the three recommended, thus precluding the determination of any potential dose-response relationship). The inclusion of only two subjects in the SCE test does not provide for meaningful statistical analysis and the influence of inter-individual variation in the SCE test was not sufficiently addressed as only pooled data was provided. The ip injection route of exposure used is not generally considered relevant to human exposure and the results of the alkaline elution assay may be due to cytotoxicity, because they were close to or in excess of the ip LD50 of glyphosate in mice. Finally, the results of the DNA oxidation damage assay may be due to cytotoxicity.

Due to the above noted limitations, the EFSA RAR (2015) concluded that the reported results do not constitute sufficient evidence to override the previously seen negative genotoxicity results and that the results are not suitable for regulatory purposes but are considered only as supplemental information.

The OCS agreed with these conclusions and noted that the data for oxidative DNA damage was not addressed in the EFSA RAR (2015). The authors reported that the oxidative DNA damage results are not surprising as the process of metabolism of pollutants and xenobiotics will generally lead to an increase in reactive oxygen species (ROS), which have a strong affinity for DNA and other cellular components. The authors concluded that the reported increased levels of 8-OhdG may represent stress due to a short-term exposure, as a part of DNA damage and repair processes. The OCS noted that the results for oxidative DNA damage (as measured by 8-OhdG) were unlikely to result in permanent DNA damage.

Chan, P & Mahler, J 1992, 'NTP technical report on the toxicity studies of Glyphosate (CAS No. 1071-83-6) Administered In Dosed Feed To F344/N Rats And B6C3F1 Mice', Toxic Rep Ser, vol. 16, pp. 1–d3.

Chan & Mahler (1992) investigated the tissue distribution, 13 repeat-dose toxicity, spermatotoxicity, mechanism of induction of salivary gland lesions and genotoxicity of glyphosate in B6C3F1 mice and F344/N rats. Only the genotoxicity studies will be discussed in this report.

Mutagenicity

The genotoxicity of glyphosate was tested using *Salmonella typhimurium* strains TA100, TA1535, TA97, and TA98 using the plate incorporation assay in the absence or presence of an exogenous source of metabolic activation Aroclor 1254-induced rat liver homogenate supernatant (S9) in rats and mice.

Glyphosate technical (0 to 10 000 µg/plate) did not induce gene mutations in *S. typhimurium* strains TA100, TA1535, TA97 or TA98 with or without metabolic activation.

Mouse peripheral blood micronucleus test

Blood smears were prepared from peripheral blood samples of dosed and control mice after termination of the 13-week toxicity study, in which glyphosate technical was administered orally to F344/N rats (n=10/sex/dose) and B6C3F1 mice (n=10/sex/dose) at 0, 3125, 6250, 12 500, 25 000, or 50 000 ppm. . Three male mice that were not included in the 13-week toxicity study were treated for four weeks with 0.2% urethane in drinking water as a positive control. Micronuclei from 10 000 normochromatic erythrocytes (NCEs) from each animal were scored. No increase in micronuclei was observed in either males or females at any dietary concentration of glyphosate technical.

The authors concluded that glyphosate does not induce genotoxicity.

Discussion and OCS conclusion

The OCS noted a number of methodological and reporting limitations to this study. The use of at least five strains of bacteria is recommended in the OECD TG 471 (1997) to detect cross-linking mutagens recommends. The study reported by Chan & Mahler (1992) included only four strains, with none detecting cross-linking mutagens. Positive controls in the *S. typhimurium* reverse mutation assay were selected according to the OECD TG 471 (1997); however; 2-aminoanthracene was used as the sole indicator of the efficacy of the S9-mix. Not all methodological details for *S. typhimurium* reverse mutation assay were provided, such as details of culture medium and culture temperature. The positive control group (quality control group) in the mouse erythrocyte micronuclei test was not treated in the same manner for the 13 weeks as other groups in the study.

The OCS considers that the above deficiencies reduce the regulatory value of the studies. However, the studies appear to follow logical scientific principles, hence the results can be used as supportive data for other studies. The OCS agrees with Chan & Mahler (1992) that the results indicate that there is no evidence that glyphosate is genotoxic.

Gasnier, C, Dumont, C, Benachour, N, Clair, E, Chagnon, MC & Seralini, GE 2009, 'Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines', Toxicology, vol. 262, pp. 184–91.

Gasnier et al. (2009) aimed to compare actions of four glyphosate-based formulations on cellular endpoints including cytotoxicity, genotoxicity and endocrine disruption in human HepG2 cells. As the endocrine disruptive potential is not considered to affect a genotoxicity endpoint, this section of the study is not included in this review. This study was critically evaluated by the EFSA RAR (2015).

Genotoxicity

The comet assay (single-cell gel electrophoresis assay, SCGE) adapted from Singh et al. (1988) was used to assess genotoxicity, with some modifications for cell preparation. Human HepG2 cells were treated with Roundup® Grands Travaux® (R400) formulation (400g/L glyphosate) for 24 hours. Experiments were repeated three times in duplicates of r 100 cells per treatment. Cell nuclei were classified as previously described (Collins 2004; Collins et al. 2008):

- 0 (undamaged)
- 1 (minimum damage)
- 2 (medium)
- 3 (maximum damage).

No genotoxicity assay results for glyphosate alone are reported in the study as glyphosate technical was used as the control material. It is unclear as to why this method was chosen. Genotoxicity results were only presented for the formulated product (R400). No explanation was provided as to why only R400 genotoxicity results are reported in the study.

Approximately 50% DNA strand breaks (25% class 1, 11% class 2 and 15.5% class 3) were observed at 5 ppm R400 treatment. At 10 ppm (24 µM glyphosate), R400 treatment caused class 2 (27%) and class 3 (36%) DNA fragments, amounting to 75% DNA damage in total. DNA fragments were reported in 35% of negative controls and the positive control (Benzo[a]pyrene) was reported to induce 95% DNA damage.

From these results, the authors concluded that DNA of the human hepatoma cell line is damaged by a glyphosate-based herbicide.

Cytotoxicity

For the cytotoxicity assays, glyphosate technical was prepared to a concentration of 360g/L and adjusted to pH 5.8 (corresponding to the concentration and pH of 2% Roundup® Bioforce® formulation) in serum free medium and diluted consecutively up to 10^{-7}. No rationale was provided by the authors for using this concentration of glyphosate in the study.

Cytotoxicity was measured with the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide; mitochondrial activity), bioluminescent ToxiLight® (cell membrane damage) and Alamar Blue® assays. Caspase 3/7 activity was measured following incubation with the glyphosate-based formulation only using the Caspase-Glo® 3/7 assay (results not described).

Initial toxicity (% of glyphosate provoking the first significant effects of ~10% toxicity) and LC_{50} for glyphosate solution was 1% and 2.78% for the Alamar Blue® assay and 1% and 1.8% for the MTT assay. An initial toxicity of greater than 2% was reported for the ToxiLight® assay.

Discussion and OCS conclusion

Only results for glyphosate technical tested alone were considered relevant for this review and are summarised here (with exception of the genotoxicity assay results). The EFSA RAR (2015) noted numerous methodological and reporting limitations (test substances not characterised, source of material for cell culture not provided, dosing concentrations not well described, serum-free media only appropriate for short-term *in vitro* exposure, incomplete data reporting, positive control data not reported), which made assessment of the results difficult. In addition, exceedingly high concentrations of glyphosate above the limit dose were used and the assays were not suitable for assessing products containing surfactants, as the potential for cytotoxic membrane disruption from surfactants is well known in *in vitro* assay systems.

Regarding the cytotoxicity and genotoxicity assay results, the OCS also noted that the authors did not provide any justification for the doses used in the cytotoxicity assays and no details were provided regarding the determination of LC_{50} and initial toxicity values for the cytotoxicity assays. These points were considered critical deficiencies, as similar assays reported significant levels of cytotoxicity (Gehin et al. 2005; Monroy et al. 2005; Prasad et al. 2009; Elie-Caille et al. 2010; Kier & Kirkland 2013; Chaufan et al. 2014).

The OCS also noted that the genotoxicity assay was performed using a glyphosate-based formulation; therefore, the results obtained cannot be fully attributed to glyphosate technical, limiting the value of the study to determine the genotoxic effect of glyphosate. No statistical analyses were performed on the genotoxicity assay results, although the authors state the effect of the formulation is concentration-dependent and that there is a 'drastic' increase in DNA damage between 5 ppm and 10 ppm R400 doses. Finally, while the authors stated that the experiments were repeated three times in duplicate for 100 cells, variation within the datasets were not reported.

Due to the numerous methodology and reporting limitations outlined above, the OCS agreed with the EFSA RAR (2015) and considered the results of Gasnier et al. (2009) not reliable for regulatory purposes.

Kier, LD & Kirkland, DJ 2013, 'Review of genotoxicity studies of glyphosate and glyphosate-based formulations', Crit Rev Toxicol, vol. 43, pp. 283–315.

Kier & Kirkland (2013) reviewed the weight-of-evidence for the genotoxic potential of glyphosate technical and glyphosate-based formulations and was discussed in the EFSA RAR (2015).

The authors discussed studies reported by Williams et al. (2000) in their review of the safety of glyphosate, aminomethylphosphonic acid (AMPA), Roundup® formulations and polyethoxylated tallow amine and conducted a literature search for articles published post-2000 with reference to glyphosate and genotoxic terms. Each article identified was evaluated to ensure it contained original results of one or more genotoxicity test on glyphosate or a glyphosate-based formulation. No monitoring studies, abstracts or sources with incomplete information were included. The scientific quality of the articles was evaluated referring to international guidelines where relevant and discrepancies were weighted. The authors noted that, in some circumstances, guideline criteria may not be met but the omission should not negatively affect the evaluation of the study, as the data were presumably collected but would not be presented individually in published papers. In other circumstances, guidelines may have greater weight in evaluating a study and failure to adhere to these guidelines would lessen the impact or reliability of the findings. Unpublished genotoxicity studies conducted for regulatory purposes were evaluated for quality in the same manner as for the identified published articles.

After scientific evaluation of the studies, including a consideration of appropriate OECD TG (OECD 471, 1997 or OECD 471, 1983), the authors concluded that glyphosate is not mutagenic to mammalian cells, does not consistently induce chromosomal effects in mammalian cells, produced negative results in the mouse micronucleus test and the majority of *in vitro* chromosomal aberration assays in the absence of metabolic activation. The authors also concluded that glyphosate-based formulations did not induce chromosomal effects *in vivo* and that glyphosate and glyphosate-based formulations do not induce aneuploidy.

The authors also concluded that evidence for genotoxicity following exposure to glyphosate-based products does not appear to be due to the glyphosate component, the positive result in cytokinesis-block micronucleus assay in cultured human epithelial cells is highly unusual and the DNA damage seen in SCE and comet assays is likely attributable to cytotoxic effects.

The authors' overall conclusion was that glyphosate and glyphosate-based products do not appear to pose significant genotoxic risk under normal human or environment exposure scenarios.

Discussion and OCS conclusion

Generally, the OCS would consider a review paper such as Kier & Kirkland (2013) to be of limited regulatory value. However, Kier & Kirkland (2013) utilised a weight-of-evidence approach to their assessment and based their analysis on both publicly available and unpublished studies, with reference to contemporary OECD TGs where appropriate. Due to the high level of analysis consistent with international standards, the OCS considers the weight-of-evidence conclusions for both glyphosate technical and glyphosate-based formulations to provide significant supportive evidence.

Li, AP & Long, TJ 1988, 'An evaluation of the genotoxic potential of glyphosate', Fundam Appl Toxicol, vol. 10, pp. 537–46.

Li & Long (1988) was considered in the EFSA RAR (2015) and investigated the genotoxic potential of glyphosate technical (98% pure).

Microbial genotoxicity

Mutagenicity was determined using three tests: the *Salmonella*/histidine reversion assay, the *E. coli* WP2 reverse mutation assay and the *B. subtilis* Rec-assay.

For the reversion assay, five histidine strains of *Salmonella typhimurium* (TA1535, TA1537, TA1538, TA98 and TA100) using the plate-incorporation assay with and without metabolic stimulation with S9 were used, as previously described (Ames et al. 1975). Glyphosate technical dissolved in distilled water was tested at 10, 50, 100, 500, 1000 and 5000 µg/plate.

The reverse mutation assay was conducted in parallel with the reversion assay with the tryptophan-*hcr* strain of *E. coli* WP2 (with and without metabolic activation with S9)

No significant induction of revertants above negative control levels or concentration-response relationships were observed.

For the Rec-assay, the standard (H17, rec+) and recombinant deficient (M45, rec–) strains of *B. subtilis* were exposed to 20, 100, 200, 500, 1000 or 2000 µg glyphosate technical, which did not inhibit growth in either strain.

Mammalian genotoxicity in vitro

Mammalian genotoxicity *in vitro* was assessed using the Chinese Hamster Ovary/ hypoxanthine-guanine phosphoribosyl transferase (CHO/HGPRT) gene mutation assay as described by Li (1985) and Li et al. (1987), and the hepatocyte/DNA repair assay previously described by Williams (1976).

Both cytotoxicity and mutagenicity were tested with and without metabolic activation.

Mutagenicity was assessed using between 2 and 25 mg/mL glyphosate technical. Treatment with any concentration of glyphosate did not significantly increase mutagenicity compared with negative controls and no significant dose-response relationship was apparent.

Significant cytotoxicity (more than 50% cell death) was observed following treatment with 22.5 mg/mL glyphosate without S9 and with 1, 2 and 10% S9, and following treatment with 17.5 mg/mL glyphosate with 10% S9. Treatment with 20 mg/mL (without S9) and 25 mg/mL (with S9) glyphosate induced significant cytotoxicity. These results indicate that treatment with high concentrations of glyphosate induces significant cytotoxicity and any positive results observed at such high concentrations should not be attributed to genotoxicity.

Non-induced primary rat hepatocytes (adult male Fischer 344 rat) were used to test the ability of glyphosate technical (12.5 ng/mL to 125 µg/mL) to induce unscheduled DNA synthesis in mammalian cells. Treatment with glyphosate did not result in cytotoxicity and no significant increase in net grains/nucleus indicative of unscheduled DNA synthesis compared with negative controls was apparent.

Mammalian genotoxicity in vivo (bone marrow chromosome aberration assay)

Genotoxicity *in vivo* was assessed as previously described (Preston et al. 1981). Glyphosate technical (1000 mg/kg bw) was administered via ip injection to Sprague-Dawley (SD) rats (18 male, 18 female). From each group, 6 rats were sacrificed at 6, 12 and 24 hours post-treatment. Chromosome aberrations were determined from 50 femoral bone marrow cells per animal (300 cells per treatment per time period). No significant increase in either chromosomal aberrations or achromatic lesions were apparent as a result of glyphosate treatment. A separate study using ^{14}C radiolabelled glyphosate confirmed that the glyphosate was able to reach the rat bone marrow (peak level at 0.5 hours, elimination half-life of more than 7.6 hours).

Discussion and OCS conclusion

No positive genotoxic responses were reported in any of the test methods performed. The authors concluded that glyphosate is not genotoxic.

The EFSA RAR (2015) noted that the *Salmonella*/histidine reversion assay results were acceptable for regulatory purposes, despite some deviations from the OECD TG 471 in that assay and *E. coli* reverse mutation assay. Specifically, it was not entirely clear what the highest glyphosate concentration was under metabolic activation in the *Salmonella*/histidine reversion assay, 2-aminoanthracene was used as the sole indicator (positive control) of S9 activation in both assays and duplicate (rather than triplicate) plating was used in both assays.

The EFSA RAR also noted that only between 5 and 20 cells were counted in the hepatocyte/DNA repair assay. The OCS noted some methodological deviations from OECD TG 475 (2014) for the mammalian *in vivo* genotoxicity chromosome aberration assay. Only one concentration of glyphosate was tested (the guidelines recommend testing a range of concentrations, from the maximum tolerated dose to a dose producing little or no toxicity) and only 50 cells per animal were examined for chromosome aberrations (in contrast to the recommended minimum 200 metaphases per animal). Furthermore, glyphosate was administered via the ip route. While this is a standard method for assessing mammalian *in vivo* chromosome aberrations, it should be noted that ip administration is not considered relevant to human exposure scenarios, so the results should be interpreted with caution.

Based on a review of the negative genotoxicity results reported by Li & Long (1988) and considering the EFSA RAR (2015) appraisal, the OCS concluded that the study was suitable for regulatory purposes.

Manas, F, Peralta, L, Raviolo, J, Ovando, HG, Weyers, A, Ugnia, L, Cid, MG, Larripa, I & Gorla, N 2009, 'Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests', Environ Toxicol Pharmacol, vol. 28, pp. 37–41.

Manas et al. (2009) was critically evaluated in the EFSA RAR (2015). Manas et al. (2009) investigated the genotoxicity of glyphosate using the SCGE (comet) assay in Hep-2 cells to determine DNA damage, as well as chromosome breakage using both the chromosomal aberration assay in human lymphocytes and the *in vivo* micronucleus test (MNT) in Balb C mice (n=5/sex/dose).

The chromosomal aberration assay was performed on lymphocytes obtained from six healthy human donors (three females and three males) were exposed to 0.2, 1.2 or 6.0 mM glyphosate technical. A known chromosomal aberration inducer (mitomycin; 0.89 µM) was used, as per OECD TG 473 (1997). The mitotic index (MI) was calculated using 2000 cells per culture and 100 metaphases were analysed for the number and type of chromosome aberrations, which were classified as per the International System of Cytogenetic Nomenclature. Two blinded observers scored each slide for chromatic breaks and gaps, chromosome breaks and gaps, dicentric chromosomes, acentric fragments and endoreduplicated cells. No significant genotoxic effects in the chromosomal aberration test were demonstrated.

Hep-2 cells were exposed to glyphosate technical (3.0, 4.5, 6.0, 7.5, 9.0, 12.0 and 15.0 mM) for 4 hours. The comet assay protocol was conducted as previously described (Singh et al. 1988), with some modifications. However, glyphosate was cytotoxic at concentrations above 7.5 mM, as assessed by the trypan blue exclusion assay—cell viability was less than 80% at 9 to 15 mM glyphosate. The results showed a significant concentration-dependent increase in DNA damage from 3.00 to 7.50 mM glyphosate technical: tail mean intensity 17- to 28-fold increase; tail length 30- to 37-fold increase; tail moment 115- to 178-fold increase).

Groups of five male and five female Balb C mice, aged 8 to 12 weeks were administered glyphosate technical (50, 100 and 200 mg/kg) via ip injection, which was repeated after 24 hours. All animals were sacrificed 24 hours after the second injection and bone marrow smears were prepared from femoral bone marrow. Bone marrow toxicity was evaluated by calculating the number of micronucleated erythrocytes (MNE) per 1000 erythrocytes and the polychromatic erythrocytes/normochromatic erythrocytes (PCE:NCE) ratio in 500 erythrocytes. There was a significant increase in MNE/analysed cells at 400 mg/kg glyphosate (3.4-fold), but not at the lower concentrations (100 and 200 mg/kg) of glyphosate and no significant change in PCE:NCE ratio at any concentration.

A single ip dose of 400 mg/kg bw glyphosate technical was administered to three groups of five mice to evaluate oxidant markers (thiobarbituric acid reactive substances; TBARs and enzymatic activities of superoxide dismutase; SOD and catalase; CAT) in liver, kidney, heart and lung. There were no consistent significant effects on TBAR, SOD and CAT levels in the liver, kidney, lung and heart.

The authors concluded that glyphosate technical is genotoxic at 400 mg/kg in mice.

Discussion and OCS conclusion

The EFSA RAR (2015) noted a number of critical methodological and reporting limitations of the Manas et al. (2009) study that either questioned the biological relevance of the results or made interpretation of the results difficult. Only 100 cells were scored per treatment in the chromosomal aberration assay, as opposed to 200 cells recommended by the OECD TG 473. The exposure route for MNT is not relevant to human exposure (ip injection) and test concentrations of glyphosate were close to the limit dose of 10 mM. Several parameters in the MNT were not reported and major deviations from OECD TG 474 were evident: erythrocytes instead of immature (polychromatic) erythrocytes were scored for micronuclei; sex of animals was not reported; independent coding of slides was not stated; blind scoring not reported. Finally, the control pH or osmolality was not reported for the comet assay and replicate data were not reported for either the chromosome assay or the comet assay.

Due to the above noted deficiencies, the OCS agreed with the conclusions of the EFSA RAR (2015) that the study results were not relevant to support the genotoxicity endpoint.

Mladinic, M, Berend, S, Vrdoljak, AL, Kopjar, N, Radic, B & Zeljezic, D 2009a, 'Evaluation of genome damage and its relation to oxidative stress induced by glyphosate in human lymphocytes in vitro', Environ Mol Mutagen, vol. 50, pp. 800–7.

Mladinic et al. (2009a) was critically evaluated in the EFSA RAR (2015) and investigated the genotoxic and oxidative potential of glyphosate technical (0.5, 2.91, 3.5, 92.8 and 580 µg/mL) in human lymphocytes with and without metabolic activation (S9). Lymphocytes were obtained from three healthy, male, non-smoking volunteers.

Genotoxicity was measured using the alkaline comet assay as previously described (Singh et al. 1988) and analysis of micronuclei and nuclear instability by applying centromere probes, as previously described (Smith et al. 2006). The results of the comet assay indicated significant genotoxic potential: increased tail length and intensity, with (1.3- and 1.4-fold, respectively) and without metabolic activation (1.1- and 1.9-fold, respectively). The authors also reported increased frequency of micronuclei (2.5-fold with metabolic activation), nuclear buds (7.3- and 4-fold, with and without metabolic activation, respectively) and nucleoplasmic bridges (32- and 8.6-fold, with and without metabolic activation, respectively).

Total antioxidant capacity was assessed by measuring Ferric-inducing ability of plasma (FRAP) and thiobabituric acid reactive substances (TBARS) activity in plasma samples. The FRAP assay measures the ability of cells to reduce Fe^{3+} to Fe^{2+}. The authors reported significant oxidative activity (total antioxidant capacity (FRAP) and lipid peroxidation (TBARS)) at 580 µg/mL glyphosate technical. These effects were generally greater with metabolic activation (S9). However, these results were presented graphically so it is difficult to determine the exact magnitude and biological relevance of the response: FRAP approximately 1.2-fold and 1.3-fold, with and without metabolic activation, respectively, compared with controls; TBARS approximately 2.3-fold and 3-fold, with and without metabolic activation, respectively, compared with controls.

No clear concentration-dependent effect was observed for any parameter. Cytotoxic effects such as the number of early apoptotic and necrotic cells were significantly increased at 580 µg/mL, with and without metabolic activation; thus any results observed at or above at 580 µg/mL should not be considered indicative of genotoxicity in this study.

The authors concluded that glyphosate technical does not pose a genotoxic or oxidative stress hazard at levels relevant for human exposure and recommended further research utilising a larger sample population.

Discussion and OCS conclusion

The EFSA RAR (2015) noted that Mladinic et al. (2009a) was a non-GLP (good laboratory practice), non-guideline study; however, the study meets broad scientific standards to determine genotoxicity and concluded (2015) that the results presented by Mladinic et al. (2009a) are acceptable.

Based on a review of Mladinic et al. (2009a) and considering the appraisal by the EFSA RAR (2015), the OCS agrees with EFSA RAR (2015) and concluded that the results of Mladinic et al. (2009a) indicate that glyphosate is not genotoxic and does not induce oxidative stress at concentrations relevant to human exposure. Furthermore, the OCS agrees with EFSA that the positive results obtained at the highest dose tested indicate a possible threshold aneugenic effect associated with cytotoxicity, rather than a DNA-reactive clastogenic effect.

Mladinic, M, Perkovic, P & Zeljezic, D 2009b, 'Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay', Toxicol Lett, vol. 189, pp. 130–7.

Mladinic et al. (2009b) was critically evaluated in the EFSA RAR (2015). Using the *in vitro* micronucleus assay, Mladinic et al. (2009b) investigated the clastogenic and aneugenic effects of glyphosate technical with and without metabolic activation (S9) on human lymphocytes obtained from three healthy, non-smoking male volunteers at 0.50, 2.91, 3.50, 92.8 or 580 µg/mL.

A significant increase in nuclear buds was reported at the highest dose tested (580 µg/mL glyphosate technical) with (approximately 3.6-fold) and without (approximately 5-fold) metabolic activation, compared with controls. Significantly increased micronuclei (approximately 2.5-fold) and nucleoplasmic bridges (approximately 3.7-fold) were reported following glyphosate treatment in the presence of metabolic activation at the highest concentration tested (580 µg/mL glyphosate technical), but no concentration-dependent increases were observed. However, these results were presented graphically, thus the exact magnitude of the reported effects at the highest concentration of glyphosate administered cannot be determined.

No effects on the cytokinesis-block proliferation index (evaluated by classifying 1000 cells as per number of nuclei as described by Surralles et al. (1995)) were evident. Significant increases for the percentage of centromere (2-fold) and 4',6-diamidino-2-phenylindole (DAPI; 2.2-fold) signals were only seen in micronuclei treated with 580 µg/mL glyphosate technical with metabolic activation (S9).

The authors concluded that glyphosate technical does adversely effect on DNA at levels of expected human exposure.

Discussion and OCS conclusion

The EFSA RAR (2015) noted some reporting and methodological deficiencies in the Mladinic et al. (2009b) report. Positive and negative control results in micronucleus assay (without S9 metabolic action) were almost indistinguishable and negative control data for nuclear buds and nucleoplasmic bridges were not reported. Furthermore, the proposed mechanism of genotoxicity is not relevant to human exposure levels: the authors state

that the estimated maximum human exposure levels correspond to acceptable safety levels based on *in vitro* endpoints; however, this is yet to be verified *in vivo*.

The OCS noted that significant and potentially biologically relevant responses to glyphosate were only observed at the highest dose tested (580 µg/mL) and no clear concentration-dependent response was demonstrated.

The OCS agreed with the conclusions of EFSA RAR (2015) that the results from Mladinic et al. (2009b) were not relevant for the genotoxicity endpoint in humans *in vivo*.

Monroy, CM, Cortes, AC, Sicard, DM & de Restrepo, HG 2005, 'Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate', Biomedica, vol. 25, pp. 335–45 (translated from Spanish)

Monroy et al. (2005) was referenced in the EFSA RAR (2015), although the complete critical appraisal was not reported. Monroy et al. (2005) investigated the cytotoxicity and genotoxicity of glyphosate (4 to 6.5 mM) in normal human cells (GM38) and human fibrosarcoma cells (HT1080).

Cytotoxicity

Concentration-dependent cytotoxicity occurred in both cell lines; however, GM38 cells were less sensitive to glyphosate technical than HT1080 fibrosarcoma cells. After exposure to 6.5 mM glyphosate technical for 4 hours, cell viability was 39% and 79% for HT1080 cells and GM38 cells, respectively. After 72 hours exposure to glyphosate technical at 3.3 mM (highest concentration for HT1080 cells) cell viability was more than 80% in GM38 cells and less than 20% in HT1080 cells.

Comet assay

Genotoxicity was investigated using the comet assay. Significant DNA damage in both cell lines when exposed for 4 hours to high concentrations of glyphosate technical was evident from the comet assay. Concentration-dependent increases in DNA migration were evident in both GM38 cells (from 1.2-fold to 5.6-fold, compared with negative control values) and HT1080 cells (negative control data not provided, so the magnitude of the response cannot be determined) (Table 3). Statistical significance was reached at 4 mM glyphosate for GM38 cells and 4.75 mM glyphosate for HT1080 cells. However, significant cytotoxicity was also apparent in HT1080 cells after 4 hours of exposure to 4.75 mM and greater concentrations of glyphosate.

Table 3: DNA migration of GM38 and HT1080 cells after 4 hours of exposure to glyphosate (reproduced from Monroy et al. (2005))

Concentration of glyphosate (mM)	Average DNA migration (µm)	% negative control
	GM38	
0 (negative control)	15.79 ± 1.05	
4	19.47 ± 1.80 *	123%
4.5	21.20 ± 1.55 *	134%
4.75	38.23 ± 2.27 *	242%

Concentration of glyphosate (mM)	Average DNA migration (µm)	% negative control
5	48.26 ± 2.39 *	305%
5.5	47.20 ± 2.94 *	299%
6.5	87.97 ± 14.1 * [c]	557%
Positive control	96.38 ± 8.85	610%
	HT1080	
4.5	27.18 ± 2.409	
4.75	29.16 ± 1.92 * [c]	
5	29.11 ± 3.19 * [c]	
5.25	27.81 ± 2.07 * [c]	
5.5	31.28 ± 2.96 * [c]	
5.75	35.87 ± 3.88 * [c]	
6	49.38 ± 11.24 * [c]	
6.5	65.84 ± 11.00 * [c]	
Positive control	110.72 ± 5.88	

* P<0.001
[c] concentration at which significant cytotoxicity is noted (cell viability < 80%)

The authors concluded that the morphology of the cells showed a concentration dependent response to treatment.

The authors concluded that the results of this study were inconsistent with other genotoxicity study results and that glyphosate technical induces single-strand DNA breaks in mammalian cells.

Discussion and OCS conclusion

The EFSA RAR (2015) noted a number of reporting and methodological deficiencies in the Monroy et al. (2005) study. The concentrations of glyphosate used were close to the limit concentration of 10 mM recommended for *in vitro* mammalian cell assays, nuclear damage was undertaken by visual scoring without coding of slides being indicated so it is unclear if investigators were blinded and results were not reported separately for replicate cultures

The EFSA RAR (2015) suggested that the concentrations used may be at the concentration threshold for cytotoxicity and there the positive results may be due to cytotoxicity. For HT1080 cells, significant cytotoxicity was observed concurrently with the significant DNA migration observations.

The OCS noted that cytotoxicity was more pronounced with increasing concentrations of glyphosate after 72 hours (data not shown) than after 4 hours. Cytotoxicity results for different exposure periods and testing concentrations demonstrated a concentration-response.

When the cytotoxicity and genotoxicity results are combined, significant cytotoxicity (as defined by the authors as less than 80% cell viability) is evident at 4.75 mM for the HT1080 cells, at which genotoxicity results should therefore no longer be considered reliable. No negative control DNA migration results were reported for the HT1080 cells. At concentrations at and below 5.5 mM, there was no significant change in the tail migration length. The DNA morphology results support this result as the percentage of DNA that was not damaged remained higher than the 'DNA damage' scores combined until 5.5 mM. In combination, these results suggest a lack of genotoxicity at concentrations not causing frank cytotoxicity.

For the GM38 cells, 80% of cells were viable at the highest concentration (6.5 mM) tested. Therefore, the data that demonstrate significant DNA migration for the GM38 cells appear reliable. The DNA migration data support the DNA morphology data, with the percentage of DNA with no damage only remaining higher than the DNA damage combined until 4 mM.

The OCS concluded that due to significant levels of cytotoxicity and the limitations noted above, the results for the HT1080 cells reported in Monroy et al. (2005) were not reliable for the purposes of conducting a risk assessment, while the genotoxicity results for the GM38 cells were considered suitable only as supporting evidence.

Prasad, S, Srivastava, S, Singh, M & Shukla, Y 2009, 'Clastogenic effects of glyphosate in bone marrow cells of swiss albino mice', J Toxicol, vol. 2009, pp. 308985.

Prasad et al. (2009) is referenced in the EFSA RAR (2015) investigated the genotoxic potential of a glyphosate-based formulation (Roundup®) by measuring the chromosomal aberrations and micronuclei in the bone marrow of Swiss albino mice.

Roundup® containing more than 41% glyphosate present as the isopropylamine salt in a soluble concentrate was used as the source of glyphosate in this study (glyphosate as referred to below represents the concentration of the product, as compared to the concentration of glyphosate technical).

Male Swiss albino mice were exposed to either 25 or 50 mg/kg bw of glyphosate (calculated from the amount of product administered) diluted in dimethyl sulfoxide (DMSO) via a single ip injection.

Chromosomal aberration assay

At 24, 48 and 72 hours following treatment, five animals from each group were terminated. The MI was calculated from 2000 femoral bone marrow cells per animal. Chromosomal aberrations were classified as breaks, fragments and exchanges. The incidence of aberrant cells was expressed as % of damaged cells (aberrant metaphases).

Glyphosate induced a significant dose-dependent and time-dependent increase in percentage incidence of aberrant cells compared to the untreated group (Table 4). Following exposure to 25 mg/kg bw glyphosate, the percentage incidence of aberrant cells increased from 3.2-fold to 4.4-fold that of negative controls between 24 and 72 hours, while exposure to 50 mg/kg bw induced an increase from 4.1-fold to 5.3-fold that of controls between 24 and 72 hours.

Micronuclei induction assay

The remaining five animals from each dose group were sacrificed at 24, 48 and 72 hours post-treatment. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was evaluated as previously described (Schmid 1975), with modifications. A minimum of 2000 erythrocytes were scored for each group and the results were presented as the number of MNPCEs/1000 PCEs. Glyphosate induced a significant dose-dependent and time-dependent increase in the frequency of MNPCEs/1000 PCEs compared to the untreated group (Table 4). Following exposure to 25 mg/kg bw glyphosate, the frequency of MNPCEs/1000 PCEs increased from 3.1-fold to 5.2-fold that of negative controls between 24 and 72 hours, while exposure to 50 mg/kg bw induced an increase from 5.5-fold to 7.2-fold that of controls between 24 and 72 hours.

Glyphosate induced a significant dose-dependent and time-dependent decrease in MI compared to the untreated group indicating cytotoxic effects on treated cells (Table 4). Following exposure to 25 mg/kg bw glyphosate, the MI decreased from 0.84-fold to 0.77-fold that of negative controls between 24 and 72 hours, while exposure to 50 mg/kg bw induced a decrease from 0.72-fold to 0.63-fold that of controls between 24 and 72 hours.

Table 4: Mutagenic activity and cytotoxic effects of glyphosate in Swiss albino mice at 24, 48 and 72 hours sampling time (n=5) (reproduced from Prasad et al. (2009))

Hours post Rx	Untreated	Positive control	25 mg/kg bw	50 mg/kg bw
	% incidence of aberrant cells (chromosome aberration)			
24	1.81 ± 0.03	12.76 ± 0.17	5.86 ± 0.12	7.46 ± 0.14
48	1.92 ± 0.03	14.35 ± 1.27	7.24 ± 0.15	8.85 ± 0.14
72	1.75 ± 0.05	15.22 ± 1.19	7.76 ± 0.4	9.24 ± 0.18
	Frequency of MNPCEs/1000 PCE's			
24	1.24 ± 0.01	15.46 ± 0.03	3.87 ± 0.02	6.86 ± 0.04
48	1.1 ± 0.01	17.5 ± 0.10	5.76 ± 0.08	8.25 ± 0.04
72	1.18 ± 0.03	18.25 ± 0.12	6.12 ± 0.07	8.48 ± 0.09
	Mitotic Index			
24	4.88 ± 0.06	2.46 ± 0.09	4.12 ± 0.05	3.54 ± 0.01
48	4.90 ± 0.02	2.12 ± 0.01	3.84 ± 0.04	3.16 ± 0.03
72	4.84 ± 0.04	1.94 ± 0.02	3.75 ± 0.03	3.06 ± 0.01

The authors concluded that the glyphosate formulation induced 'chromosome aberrations and micronuclei induction accompanied by inhibition of cell proliferation in Swiss albino mice following ip administration'.

Discussion and OCS Conclusion

The EFSA RAR noted one critical methodological limitation in Prasad et al. (2009). DMSO is an unusual vehicle for use in *in vivo* genotoxicity studies, particularly for glyphosate, which is water soluble. The use of DMSO as a vehicle by the ip route has been shown to dramatically enhance the toxicity of a glyphosate-based formulation, thus DMSO as a vehicle for administration of glyphosate-based formulations via the ip route may not be relevant to normal human exposure

The OCS noted a number of additional methodological limitations. Test animals were exposed to Roundup® (formulated product) rather than glyphosate technical. The effect of glyphosate technical in isolation was not investigated. The use of benzo(a)pyrene without metabolic activation is not currently considered an acceptable positive control (OECD TG 475, 2014); however, at the time of the study being conducted the only criteria for the positive control was that it should be known to induce chromosome aberrations *in vivo*. Intraperitoneal injection is not considered a relevant human exposure route by contemporary standards (OECD TG 475, 2014); however, at the time of the study being conducted the guidance stated that it was an acceptable exposure route. In addition, the OCS noted a number of reporting shortcomings in the study (there was no indication of historical control data, clinical observations, such as food and water intake, body weight data etc were not reported, the dose concentrations used were not justified and the MI was given as a mean of the group and individual results were not reported).

Following consideration of the EFSA RAR and the above deficiencies, the OCS considered that a conclusion regarding the genotoxicity potential of glyphosate technical could not be drawn from this study. The OCS considered that the results of the study were not informative in regards to the genotoxic effects of glyphosate technical. The use of DMSO as a vehicle for administration rendered the study inadequate for assessing the genotoxic potential of a glyphosate-based formulation via the ip route, as it may produce toxic effects that are not relevant for normal human exposure.

Rank, J, Jensen, AG, Skov, B, Pedersen, LH & Jensen, K 1993, 'Genotoxicity testing of the herbicide Roundup® and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test', Mutat Res, vol. 300, pp. 29–36.

Rank et al. (1993) was evaluated in the EFSA RAR (2013). Rank et al. (1993) investigated the genotoxicity of Roundup® and glyphosate technical using the mouse bone marrow micronucleus assay (as described by Schmid (1975)), and the *Salmonella* plate incorporation assay, as previously described (Ames et al. 1975; Maron & Ames 1983) using Roundup® only.

Groups of 7–10 mice (male and female) were terminated at 24 and 48 hours post-treatment (glyphosate technical 100, 150 or 200 mg/kg bw) or 24 hours post-treatment (Roundup® 133 or 200 mg/kg bw) via ip administration. The frequency of MNPCEs was evaluated as previously described (Schmid 1975), with modifications. A minimum of 1000 PCEs were examined to determine the frequency of MNPCEs. No genotoxic effects of either glyphosate or Roundup® were evident from the mouse bone marrow micronucleus assay.

The *Salmonella* plate incorporation assay was performed using two of the five strains recommended by the OECD TG 471 (1997): TA98 and TA100. The mutagenicity of Roundup® was determined with and without metabolic activation (S9). A weak mutagenic effect was demonstrated at 360 µg/plate in TA98 without metabolic activation (4-fold) and at 720 µg/plate in TA100 with metabolic activation (3.2-fold).

The authors concluded that Roundup® (but not glyphosate technical) induces weak mutations in *S. typhimurium*.

Discussion and OCS conclusion

The EFSA RAR (2015) noted that while the results showed spontaneous increases in the mutation rate, no clear dose-response was observed. In addition, there was marked cytotoxicity at 360 or 720 µg/plate onwards, which limited a reliable assessment of the mutagenicity results. Finally, the plate number scored was inconsistent in the *Salmonella* plate incorporation assay.

The EFSA RAR (2015) noted that the results for the micronucleus test (indicating dose-related cytotoxicity of the Roundup® formulation, which is not observed with glyphosate technical treatment) are consistent with previous results, but that a reliable assessment of the plate incorporation assay was not possible due to cytotoxicity.

The OCS agreed with this appraisal and noted some addition deviations from OECD TG 471 for the *Salmonella* plate incorporation assay. Specifically, only two (of five recommended) bacterial strains were tested, only four (of five recommended) concentrations of Roundup® were used and glyphosate technical was not tested. Therefore, the genotoxicity of glyphosate technical could not be assessed and interpretation of the results obtained using Roundup® was difficult.

4.2 Oxidative stress

Astiz, M, de Alaniz, MJ & Marra, CA 2009, 'Effect of pesticides on cell survival in liver and brain rat tissues', Ecotoxicol Environ Saf, vol. 72, pp. 2025–32.

Astiz et al. (2009) was critically evaluated in the EFSA RAR (2015). Cellular and DNA damage in the liver and brain were assessed following ip administration of 10 mg/kg bw glyphosate either alone or in combination (with dimethoate and/or zineb) to male Wistar rats three times per week for five weeks. The focus of this study was to determine whether oxidative damage induced by pesticides would be capable of inducing sufficient damage to result in cell death (a known hallmark of neurodegenerative diseases).

Caspase and calpain activity

Caspase-3 activity in samples of liver and brain (substantia nigra and cerebral cortex) was determined using a colorimetric assay kit (CASP-3-C). Milli- (m) and micro- (µ) calpain activities were measured as previously described.

Caspase-3 activity in liver and brain was unaffected by glyphosate, both alone and in combination. Glyphosate alone and in combination did not significantly affect activity of m-calpain in liver; however, glyphosate (alone) significantly increased m-caplain activity in brain tissues (1.2- and 1.3-fold in the cerebral cortex and substantia nigra, respectively, compared with controls). The combination of glyphosate with zineb significantly increased m-calpain activity in the substantia nigra region of the brain (1.3-fold), while the combination of glyphosate with

dimethoate (1.1-fold in the cerebral cortex and 1.6-fold in the substantia nigra) and the combination of all three chemicals (1.1-fold in the cerebral cortex and 1.8-fold in the substantia nigra) significantly increased m-calpain activity in both regions of the brain. The authors stated that 'similar results were obtained for μ-calpain activity'; however, the data were not presented in the publication.

Integrity of inner and outer mitochondrial membranes

The integrity of the inner mitochondrial membrane was assessed by testing the electrochemical proton gradient with a membrane potential-sensitive probe (JC-1) as described previously (Reers et al. 1991). Glyphosate alone did not significantly reduce inner membrane potential. In the liver, glyphosate in combination with either zineb (to approximately 75% of control) or dimethoate (to approximately 55% of control) or both (to approximately 35% of control) significantly reduced inner membrane potential. The combination of glyphosate and dimethoate (to approximately 45% and 50% of controls in the substantia nigra and cerebral cortex, respectively) and the combination of all three chemicals (to approximately 30% and 40% of controls in the substantia nigra and cerebral cortex, respectively) significantly reduced inner membrane potential in both regions of the brain.

The integrity of the outer mitochondrial membrane was assessed by determining cytochrome *c* (Cyt_c) oxidase activity (indicative of apoptosis), in the presence and absence of a detergent. Glyphosate did not reduce outer membrane potential in any tissue alone or in combination with either zineb or dimethoate. Outer membrane potential in the substantia nigra region of the brain was significantly reduced by the combination of all three chemicals (to approximately 75% of control).

However, the results for the effects of the various chemicals on inner and outer mitochondrial membranes was presented graphically, thus the exact magnitudes of the reported changes cannot be accurately determined.

DNA fragmentation

DNA was obtained from liver and brain and fragmentation patterns were analysed using the DNA laddering technique previously described (Sambrook & Fritsch 1989). The authors reported a typical, but not apoptotic pattern of DNA damage, which was more evident when pesticides were administered in combination (results of agarose gel electrophoresis presented visually, with no quantifiable results available).

Mitochondrial cardiolipin content

Glyphosate alone significantly reduced mitochondrial cardiolipin content in brain tissues (by approximately 0.46-fold and 0.57-fold of controls in the substantia nigra and cerebral cortex, respectively) but not liver. Mitochondrial cardiolipin content was also significantly reduced by the combination of glyphosate with zineb (liver: by approximately 0.31-fold; substantia nigra: by approximately 0.44-fold; cerebral cortex: by approximately 0.55-fold, compared with controls) or dimethoate (substantia nigra: by approximately 0.54-fold; cerebral cortex: by approximately 0.66-fold, compared with controls) or both (liver: by approximately 0.51-fold, substantia nigra: by approximately 0.6-fold; cerebral cortex: approximately 0.64-fold, compared with controls).

Conclusions

The study authors concluded that agrochemicals in combination produce more damage indicative of oxidative stress than agrochemicals alone and that the agrochemical–induced oxidative stress condition modifies the biomarkers of redox status in liver and brain tissues that may be linked to cell death. They further concluded that

damage indicative of oxidative stress was greatest in the midbrain substantia nigra and the agrochemical–induced oxidative stress condition could be responsible for calpain activation.

Discussion and OCS Conclusion

The EFSA RAR (2015) noted a number of critical methodological deficiencies in Astiz et al. (2009). The very small test groups of only one sex (n=4 males) weakened the validity of the results. No significant effects were reported for liver endpoints, despite its close proximity to the administration site, and the effects on the brain are not biologically plausible for humans due to the blood-brain barrier and rapid elimination kinetics of glyphosate via urine. They further noted a number of reporting deficiencies (no information on test substance purity, animal husbandry, clinical observations, food and water intake, body weight data).

The OCS agrees with the conclusion of the EFSA RAR (2015) that the study results were not relevant for the genotoxicity endpoint. In contrast, the IARC monograph interpreted these results as supportive of the oxidative stress mechanism of action for carcinogenicity.

Chaufan, G., Coalova, I., & de Molina, M. D. C. R. (2014): Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells: Differences with its active ingredient. International Journal of Toxicology, 33(1), 29–38.

The multi-component study by Chaufan et al. (2014) sought to explore possible mechanisms for glyphosate cytotoxicity in a human hepatoma cell line (HEPG2) and was considered by the EFSA RAR addendum (2015) but a detailed critique of the study was not provided. Cells were exposed to either glyphosate technical (95% pure), AMPA or a commercially available glyphosate-based formulation (Roundup Ultra® Max; 74% w/w glyphosate ammonium salt equivalent to 67.9% glyphosate acid with 25.3% adjuvants and inert substances; soluble granule formulation).

Cell viability

To determine cell viability, the conversion of MTT to blue formazan crystals within the mitochondria of viable cells was measured using colorimetric detection. Cells were exposed for 24, 48 and 72 hours to glyphosate technical (0 to approximately 0.95 mg/mL), AMPA (0 to approximately 0.95 mg/mL) or glyphosate-based formulation (0 to approximately 0.05 mg/mL).

At concentrations below approximately 0.95 mg/mL (from the published graph), glyphosate technical and AMPA had no significant effect on cytotoxicity at 24, 48 or 72 hours (LC_{50} > 0.90 mg/mL). In contrast, the glyphosate formulation caused a time- and concentration-dependent decrease in cell viability at concentrations below 0.05 mg/mL, thus any positive results reported following exposure to the glyphosate formulation at or above 0.05 mg/L should not be considered indicative of genotoxicity. However, it was not clear from the publication what concentrations of test substances were used in each experiment, thus the biological releance of the results is questionable. The estimated glyphosate formulation LC_{50} values for 24, 48 and 72 hours were 0.041, 0.035 and 0.035 mg/mL, respectively.

Antioxidant enzyme activity

Cells were grown at confluence with the different treatments for 24 hours at concentrations where cell viability was maintained at greater than 80%. Activity of CAT (Aebi 1984), glutathione-S-transferase (GST) (Habig et al. 1976) and SOD (Beauchamp & Fridovich 1971) in the cell supernatants were measured using previously published methods. Glyphosate technical, AMPA and the glyphosate formulation had no effect on the activity of antioxidant enzymes CAT or GST compared to controls (Table 5).

Exposure to glyphosate formulation (but not glyphosate technical or AMPA) caused a statistically significant and biologically relevant increase in SOD activity (approximately 1.7-fold greater than control; $p < 0.05$) (Table 5).

Glutathione (GSH) content

The study authors reported that GSH concentration was measured as described by Anderson (1985). However, the exact methodology used to determine GSH concentration is unclear and could not be validated, as the authors later refer to another publication (Gasnier et al. 2009) for determination of GSH concentrations, which does not contain methodology to measure GSH levels.

Cells were incubated at early confluence (80–90% confluent) with the different treatments. However, the incubation period was not specified and concentrations of specific treatments (glyphosate-based formulation, glyphosate technical and AMPA) were not clearly reported].

Exposure to AMPA and glyphosate formulation but not glyphosate technical, caused a statistically significant and biologically relevant increase in GSH (approximately 1.8- and 1.9-fold greater than control, respectively; $p < 0.001$) (Table 5).

Table 5: Enzymatic (SOD, CAT, GST) and non-enzymatic (GSH) antioxidant capacity of HEPG2 cells following 24 hour exposure to test substance from five independent experiments (reproduced from Chaufan et al. (2014)).

Test substance	SOD activity (% control ± standard deviation)	CAT activity (% control ± standard deviation)	GST activity (% control ± standard deviation)	GSH content (% control ± standard deviation)
Control	100.0 ± 7.8	100.0 ± 39.9	100.0 ± 9.4	100.0 ± 14.0
Glyphosate technical	128.7 ± 17.3	88.1 ± 23.5	110.2 ± 25.3	123.6 ± 23.5
AMPA	148.2 ± 37.0	100.9 ± 23.0	121.4 ± 19.2	181.5 ± 31.1**
Glyphosate formulation	173.7 ± 66.6*	79.1 ± 15.1	82.0 ± 19.5	188.9 ± 36.3**

*Significantly different to control ($p < 0.05$)

**Significantly different to control ($p < 0.01$)

SOD: superoxide dismutase; CAT: catalase; GST: glutathione-S-transferase; GSH: glutathione; AMPA: aminomethylphosphonic acid.

Reactive oxygen species (ROS)

To measure ROS formation, a cell-permeable non-fluorescent probe (2′,7′-dichlorodihydrofluorescein diacetate; H_2DCFDA) was used. This probe is hydrolysed by cellular esterases to form 2′,7′-dichlorodihydrofluorescein (H_2DCF) and retained within the cell. In the presence of ROS and cellular peroxidases, the retained non-fluorescent H_2DCF probe is transformed to a the highly fluorescent 2′,7′-dichlorofluorescein, which can be visualised at 525 nm when excited at 488 nm.

Cells were incubated with glyphosate technical (0.9 mg/mL), AMPA (0.9 mg/mL) or glyphosate-based formulation (0.04 mg/mL) for 24 hours. The LC_{50} was used for glyphosate-based formulation testing as increased ROS production was observed at this concentration. The LC_{20} concentration (900 mg/L) was used for glyphosate and AMPA testing.

Cytoplasmic ROS formation was measured by exposing cells to glyphosate formulation (concentration not specified).

No significant difference in ROS formation was observed in cells treated with glyphosate technical or AMPA. A significant increase in ROS formation was observed in cells treated with glyphosate formulation (approximately 1.4-fold greater than control), which was also apparent by fluorescence microscopy as a significant increase in the presence of intracellular ROS compared to control.

Tyrosine nitration

Neither glyphosate technical nor AMPA treatment resulted in an increase in tyrosine nitration compared to controls. A significant increase in tyrosine nitration was observed following treatment with glyphosate formulation (approximately 1.4-fold greater than control; $p < 0.01$).

Nuclear morphology assessment

Visualisation of cells in an apoptotic state (regulated cell death) was performed by assessing cell nuclear morphology.

Cells treated with glyphosate formulation showed a significantly higher percentage of condensed and fragmented nuclei compared to controls (approximately 1.2-fold greater; $p < 0.01$), which the authors conclude was indicative of apoptotic cell death. No positive control data was provided.

Caspase activity

Caspases participate in the programmed cell death pathway; in contrast to necrotic cells, some apoptotic cells display caspase 3/7 activity. Caspase activity was measured by indirectly measuring the level of caspase 3/7 activity in cell lysates.

Caspases 3/7 were significantly activated by exposure to the glyphosate formulation. Glyphosate technical or AMPA treatment did not result in changes in caspase 3/7 activity compared to controls (data not shown). On the basis of these results, the authors suggested that the apoptotic response to treatment with glyphosate formulation is mediated via a caspase-dependent apoptotic pathway.

Conclusions

The authors concluded that glyphosate formulations induce cytotoxicity, ROS production, antioxidant defence induction and apoptosis and that they contain excipients that, combined with glyphosate induce toxic effects, which are not observed with glyphosate alone.

Discussion and OCS Conclusion

The relevance of this study to the Australian context is limited. Roundup Ultra® Max (Sinochem, Australia) is available on the Australian market as 570 g/L (57.0% w/v) glyphosate present as the potassium salt in a liquid formulation. The glyphosate-based formulation used in the study, Roundup Ultra® Max (Monsanto, Argentina), was 74.7% w/w glyphosate present as the ammonium salt in the soluble granule formulation (and prepared as per directions in water) and it is not known if the formulation excipients are the same.

The cytotoxicity of the glyphosate formulation was significantly higher than glyphosate technical or AMPA. In order to maintain 80% cell viability throughout all the experiments, a much lower concentration of glyphosate formulation was used (and therefore glyphosate concentration) than for glyphosate technical or AMPA. The cell responses between treatments are therefore not directly comparable.

There are a number of additional methodological uncertainties that limit the usefulness of this study for regulatory purposes and the conclusions that can be drawn from the results. There was no consideration of the impact of the lack of protein source (which was included in all other experiments) and pH changes for up to 72 hours in the cell viability experiment (eg the viability of control cells under those conditions was not provided). This is most significant for glyphosate technical and AMPA where no differences to controls were observed in the assays (potential for a false negative result). The use of the LC_{50} for the glyphosate formulation treatment condition in order to increase ROS formation and LC_{20} for the other treatments questions the validity and comparability of the results.

In addition, there were a number of reporting deficiencies that make interpretation of the results difficult. Concentrations of treatments were not specified for each experiment (antioxidant enzyme activity, GSH, nuclear morphology and caspase assays) and the methodology for the GSH determinations was cited as being described in another published article, which was not contained in that referenced article and not specified within the current article. The concentration of glyphosate in the test preparation of the formulated product is unclear. Finally, control cell data was not provided and therefore the basal cytotoxicity results for glyphosate technical and AMPA cannot be confirmed at 0.9 mg/mL after 27 hours. The lack of clarity surrounding concentrations of test substances reduces confidence in the biological relevance of the results, as it is not clear whether these concentrations are relevant to realistic human exposure.

In addition, there was a large amount of variability in SOD activity for the glyphosate formulation (standard deviation 66% vs 7.8–37% for other conditions).

Taking into account uncertainties in the study and assuming that the same concentration of glyphosate formulation (0.04 mg/mL) was used for all experiments, unless otherwise specified, the OCS considered Roundup Ultra® Max (soluble granule formulation) containing 74.7% glyphosate ammonium salt to be highly cytotoxic *in vitro* (LC_{50} = 0.04 mg/mL product at 24 hours), a stimulator of increased ROS production *in vitro*, a stimulator of tyrosine nitration *in vitro*, and a stimulator of apoptosis (based on nuclear morphology changes and increased caspase 3/7) *in vitro*.

Therefore, based on the findings of Chaufan et al. (2014), the OCS considered oxidative stress and apoptosis a plausible mechanism of action for the *in vitro* cytotoxicity of the glyphosate formulation.

Elie-Caille, C, Heu, C, Guyon, C & Nicod, L 2010, 'Morphological damages of a glyphosate-treated human keratinocyte cell line revealed by a micro- to nanoscale microscopic investigation', Cell Biol Toxicol, vol. 26, pp. 331–9.

Elie-Caille et al. (2010) examined the possible cytotoxic mechanisms of glyphosate *in vitro* by examining cell viability, morphology, surface topography and intracellular structures following glyphosate exposure. This study was cited briefly in the EFSA RAR (2015); however, no detailed critique was presented.

Cell viability

Human keratinocyte HaCaT cells were exposed to 0, 10, 18, 20, 25, 28, 30, 35, 40, 45, 50, 55, 60 or 70 mM glyphosate technical (powder, ≥ 95% purity; equivalent to 0, 1.7, 3.0, 3.4, 4.2, 4.7, 5.1, 5.9, 6.8, 7.6, 8.5, 9.3, 10.1 or 11.8 mg/mL) for 0.5, 4, 18 and 24 hours. Cell viability was measured using an MTT assay.

HaCaT cells displayed time- and concentration-dependent cytotoxicity (indicating decreased cell viability) following treatment with glyphosate technical for 30 minutes to 18 hours. Little cytotoxicity was observed at 30 minute incubation times at ≤ 40 mM glyphosate technical, with concentration-dependent decreases in cell viability observed at higher concentrations. Cytotoxicity was observed at ≥ 10 mM (lowest dose) at 4, 18 and 24 hours post-treatment. The dose-response curves following treatment with glyphosate technical for 18 and 24 hours were very similar. The IC_{50} values were 53, 28 and 20 mM following 30 minutes, 4 hours and 18/24 hours incubation with glyphosate technical, respectively.

ROS production

Cells were incubated for 30 minutes with 50 mM glyphosate (IC_{50}; equivalent to 8.5 mg/mL glyphosate) and hydrogen peroxide (H_2O_2) was visualised using a previously validated method (Carini et al. 2000).

The authors stated that H_2O_2 was increased in cells treated with 50 mM glyphosate for 30 minutes, as evidenced by increased and more diffuse fluorescence (and therefore H_2O_2) in the cytosol. In contrast, fluorescence was concentrated as a 'halo' around the nucleus in control cells. However, no quantifiable data were presented.

Structural disorders

For tubulin visualisation, cells were incubated with 0, 21 mM (IC_{50}) or 25 mM (IC_{65}) glyphosate (equivalent to 3.6 and 4.2 mg/mL glyphosate, respectively) for 18 hours. For cell structural visualisation, cells were incubated for 18 hours with 25 mM glyphosate (IC_{65}) or for 30 minutes with 50 mM glyphosate (IC_{50}).

At the IC_{50} (50 mM glyphosate, 30 minutes), cytoskeletal staining was less confined to cells, less spread than controls and had an elongated morphology. In cells treated at the IC_{65} (25 mM glyphosate, 18 hours), weak tubulin staining throughout the entire sample indicated severely reduced cell integrity.

The authors postulated that a clean circular area of 30 µm in diameter may correspond to the print of a removed cell as a consequence of glyphosate treatment and that accumulated materials on a 20–30 µm delimited substrate area may correspond to cell remains after treatment with highly concentrated glyphosate.

Cell membranes were flattened in treated cells, which was related to an alteration of the regular distribution of native protrusions that characterised control cells.

Cells treated with glyphosate were two-fold less numerous than control cells and were replaced by elongated shapes similar to half-confluent cultures. The cell size was reduced following treatment with glyphosate (IC_{50}, 50 mM glyphosate, 30 minutes).

The authors reported DNA condensation and fragmentation via staining with DAPI but these are not presented in the results (except as the figure of DAPI-stained cells).

The authors concluded that glyphosate induces oxidative stress in HaCaT cells leading to cell destruction via an apoptotic pathway. The authors stated that exposing human epithelial HaCaT cells to glyphosate technical induced morphological modifications (shrunk, elongated cell shapes with significantly affected cell adhesion potential), indicative of apoptosis. However, the authors stressed that the spontaneously transformed, immortalised human epithelial HaCaT cell line derived from adult skin used in these experiments exhibit possible distinct functional deficiencies compared with normal human keratinocytes; thus, the results of these experiments cannot be directly extrapolated to *in vivo* keratinocyte behaviour in response to glyphosate exposure.

Discussion and OCS Conclusion

There are no OECD test guidelines that guide assessment of oxidative stress. Two of the few guidelines relating to *in vitro* genotoxicity testing (OECD TG 473, 2014 and 476, 2015) recommend care in interpreting DNA damage results where changes in pH or osmolality and excessive cytotoxicity are present as these can lead to false positives. It is recommended that determinations be made at high, medium and minimal cytotoxicity.

The concentrations of glyphosate used in this experiment are high for an *in vitro* experiment (≥ 10 mM). Given that the experiments were performed at the IC_{50}, cell responses due to osmotic stress rather than glyphosate toxicity cannot be excluded.

The EFSA RAR noted that while the authors concluded that treatment with glyphosate (50 mM) for 30 minutes resulted in overproduction of H_2O_2, determined as a thicker and more intense fluorescent area, no quantitative estimate is available.

The OCS also noted some reporting deficiencies. For example, light microscopy images of the cells for the ROS assay were not included. Although the fluorescence within treated cells was more intense, determinations regarding distribution within the cell cannot be made without light microscopy images. In the ROS/H_2O_2 experiment, it is not clear why the authors permeabilised and blocked the cells following glyphosate treatment as no other treatments or staining followed.

In addition, a number of methodological limitations were noted by the OCS. Cells were incubated with H_2DCFDA and washed extensively prior to glyphosate treatment. As H_2DCFDA is cell-permeable, the fluorescence observed is more likely to be a result of ROS present in the cells prior to treatment. Given cell structural changes were observed in the presence of the glyphosate treatment at the IC_{50}, the increased fluorescence and change in distribution may be an artefact of the change in treatment-induced cell shape after H_2DCFDA staining has already occurred. Light microscopy images were not provided to accompany the confocal microscopy images of the tubulin and DAPI stained cells; as per the ROS experiment, it is difficult to assess re-distribution of the cytoskeleton in the absence of these images.

With regard to cell structural changes, the authors themselves identify that the IC_{50} leads to a two-fold reduction in the number of cells. On the basis of the information presented, it is difficult to conclude that glyphosate induces structural cellular changes, as it is possible that sub-confluent cells may also inherently develop abnormal cell morphology in the absence of glyphosate treatment due to the reduction in cell numbers.

Gehin, A, Guillaume, YC, Millet, J, Guyon, C & Nicod, L 2005, 'Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach', Int J Pharm, vol. 288, pp. 219–26.

Gehin et al. (2005) examined and compared the cytotoxicity of glyphosate alone and as a formulated product (Roundup 3 plus®) on the immortalised human keratinocyte cell line HaCaT and investigated the potential protective effects of additional antioxidants such as Vitamin C and Vitamin E on toxicity *in vitro*. This study was considered in the EFSA RAR addendum (2015) but a detailed critique was not presented.

Cytotoxicity

Human HaCaT cells were incubated in 0, 100 or 200 µM Vitamin C, Vitamin E or a combination of both for 0, 24, or 48 hours. Following treatment with antioxidants, cells were incubated overnight in 0, 10, 12.5, 15, 17.5, 20, 22 and 25 mM glyphosate or Roundup 3 plus® with or without Vitamin E (100 or 200 µM) and/or Vitamin C (100 or 200 µM).

The authors used chemometrics (combination of mathematical and statistical methods), a matrix-based approach to study simultaneous variations of several experimental conditions, which reduces the number of experimental assays required. The toxicity of glyphosate and Roundup 3 plus® was quantified by calculating the IC_{50} and the slope of each of the treatment conditions.

Roundup 3 plus® had a direct effect on the IC_{50} and the slope of the curve, and was more toxic on HaCaT cells than glyphosate alone. The measured IC_{50} and slope values were 22 and -2.8 mM respectively for glyphosate technical; and 19.5 and -7.2 mM respectively for Roundup 3 plus® (statistical significance not indicated).
The authors concluded that this indicated that Roundup 3 plus® was more toxic on epidermal cells than glyphosate technical.

The authors noted that these effects could be due to the inclusion of surfactants and other compounds in Roundup 3 plus® and postulated that these additional compounds could enhance penetration of glyphosate to the epidermal cells and increase the kinetic of intracellular passage resulting in cytotoxicity.

The authors concluded that glyphosate-based formulations may promote oxidative damage to human epidermal cells. However, these findings were only based on cell viability results and not assays specific for oxidative damage.

The authors concluded that vitamins C and E provided a level of protection against the cytotoxic effects of glyphosate or Roundup 3 plus®. No positive control was used to demonstrate the assay sensitivity, hence it was difficult to determine the relevance of the small changes in slope and IC_{50}.

The authors concluded that the toxic effects of glyphosate and glyphosate-based formulations in human skin can be reduced by adding antioxidant compounds in formulations. Treatment with Vitamin C and E alone appeared to be more protective than combining Vitamin C and E and the activities of Vitamin C and Vitamin E were similar.

The study also reported that a pre-incubation period with antioxidants did not modify the cytoprotection compared to simultaneous glyphosate-antioxidant co-incubation.

The authors reported high reproducibility and good stability of the modelling used, as indicated by the variance coefficient values of less than 4% in most cases. The fitting of the model to the results gave IC_{50} and the slope values of 0.987 and 0.988, respectively.

Discussion and OCS conclusions

The OCS noted some methodological limitations in this study. The study only used the MTT assay to measure cell viability through measurement of cellular metabolic activity. Thus, a decrease in cellular metabolic activity due to glyphosate or glyphosate-based formulation treatment could lead to the cells being misclassified as unviable. The authors suggested that the mechanism of glyphosate toxicity is through the induction of oxidative stress. However, the results were not validated using other assays to specifically measure oxidative stress within the cells. Other mechanisms of glyphosate- or glyphosate-based formulation-induced toxicity cannot be ruled out.

The OCS also noted that no statistical results were reported. Further, no positive controls were employed to determine the level of significance of the changes reported.

The OCS concluded that the relevance of this *in vitro* study to the *in vivo* situation is unclear, as providing vitamins in a cell culture solution does not reflect systemic human exposure scenarios.

Kwiatkowska, M, Huras, B & Bukowska, B 2014, 'The effect of metabolites and impurities of glyphosate on human erythrocytes (in vitro)', Pestic Biochem Physiol, vol. 109, pp. 34–43.

Kwiatkowska et al. (2014) investigated the toxicity of glyphosate technical, its metabolites (AMPA, methylphosphonic acid) and its impurities (PMIDA, N-methylglyphosate, hydroxymethylphosphonic acid, bis-(phosphonomethyl)amine) on haemolysis, haemoglobin oxidation, ROS formation and morphology of human erythrocytes. This paper was considered by the EFSA RAR (2015) but no detailed critique was provided.

Erythrocytes were isolated from four to five healthy human donors. Cells were incubated with the test substances (0, 0.01, 0.05, 0.1, 0.25, 0.5 or 5 mM) for 1, 4 or 24 hours.

Cell viability

Haemolysis (released haemoglobin) was < 2% of the positive control for all test substances. Haemolysis was significantly increased at biologically relevant magnitudes following incubation for one hour with glyphosate technical (1.8-fold), its impurities (*N*-methylglyphosate and hydromethylo-phosphonic acid: 2.4-fold; bis-(phosphonomethyl)amine: 1.7-fold) and metabolites (AMPA: 1.8-fold; methylphosphonic acid: 1.9-fold)) at 5 mM and for PMIDA (1.6-fold) at ≥ 0.5 mM.

Haemolysis was significantly increased at biologically relevant magnitudes following incubation for 4 hours with glyphosate technical (1.6-fold), AMPA (2.2-fold), methylphosphonic acid (2.1-fold) and hydroxymethyl-phosphonic acid (2.2-fold) at 5 mM and for PMIDA (1.6- to 2.4-fold), *N*-methylglyphosate (1.8- to 2.4-fold) and bis-(phosphonomethyl)amine (1.7- to 2.3-fold) at ≥ 0.5 mM.

Haemolysis was significantly increased at biologically relevant magnitudes following incubation for 24 hours with glyphosate technical (1.6- to 1.7-fold), *N*-methylglyphosate (1.9- to 2.3-fold) and hydroxymethyl-phosphonic acid (1.5- to 2-fold) at ≥ 0.25 mM, for methylphosphonic acid (2- to 2.4-fold), PMIDA (1.5- to 2.1-fold) or bis-(phosphonomethyl)amine (1.8- to 2.3-fold) at ≥ 0.10 mM and for AMPA (1.5- to 2-fold) at ≥ 0.05 mM.

Small (and possibly biologically irrelevant) but significant increases in methaemoglobin (met-Hb) were observed following one hour incubation with AMPA (1.3-fold), methylphosphonic acid (1.2-fold), hydroxymethyl-phosphonic acid (1.3-fold) and bis-(phosphonomethyl)amine (1.4-fold) at ≥ 0.5 mM, *N*-methylglyphosate at ≥ 0.5 mM (1.2- to 1.3-fold) and PMIDA at ≥ 0.1 mM ()1.2- to 1.3-fold, but not glyphosate technical.

Small (and possibly biologically irrelevant) but significantly increased met-Hb concentrations were observed following 4 hours incubation with glyphosate technical (1.3-fold), AMPA (1.3-fold) and bis-(phosphonomethyl)amine (1.4-fold) at ≥ 5 mM, methylphosphonic acid (1.2- to 1.4-fold), *N*-methylglyphosate (1.2- to 1.4-fold) and hydroxymethyl-phosphonic acid (1.2- to 1.5-fold) at ≥ 0.5 mM and PMIDA at ≥ 0.05 mM (1.3- to 1.4-fold).

Biologically relevant, significant increases in met-Hb concentrations were observed following 24 hours incubation with methylphosphonic acid (1.3- to 1.4-fold), *N*-methylglyphosate (1.4- to 1.5-fold), hydroxymethyl-phosphonic acid (1.4- to 1.5-fold), bis-(phosphonomethyl)amine (1.5- to 1.8-fold) at ≥ 0.5 mM, for AMPA at ≥ 0.25 mM (1.4- to 1.5-fold), glyphosate technical at ≥ 0.1 mM (1.2- to 1.8-fold) and PMIDA at ≥ 0.05 mM (1.2- to 1.4-fold).

ROS production

Significantly increased ROS production was observed following exposure to *N*-methylglyphosate at ≥ 0.5 mM and glyphosate technical, its metabolites and impurities (except *N*-methylglyphosate) at ≥ 0.25 mM. However, the results were provided graphically without actual data, hence is the biological relevance of these results are difficult to confirm. The largest increase in relative ROS production was for 5 mM bis-(phosphonomethyl)amine, which was much greater than for the other compounds at the same concentration. The increases for other compounds at the same concentration were comparable. The presence of an antioxidant, *N*-acetylcysteine (NAC), significantly reduced the ROS increasing effect of all compounds at all concentrations.

Erythrocyte morphology

There was no significant difference in cell size (forward scatter pulse area; FSC-A) following any of the treatments. Cells treated with 5 mM bis-(phosphonomethyl)amine exhibited a small (less than 10% and therefore possibly not biologically relevant) but significant difference in cell granularity (lower side scatter pulse area; SSC-A) but there was no significant effect following treatment with any other compound. Again, the results were provided graphically without actual data. The effect of 5 mM bis-(phosphonomethyl)amine was observed by phase contrast microscopy as the formation of echinocytes on the erythrocytes.

Conclusion

The authors concluded that the compounds tested were not haemolytic, nor did they increase met-Hb levels (haemolysis was negligible at < 2% and met-Hb production was within physiological values).

Increased ROS production was observed for glyphosate technical, its metabolites and impurities at levels consistent with acute poisoning, which the authors concluded was suggestive of an oxidative stress mode of action

at these levels. Glyphosate technical, its metabolites and impurities (except for bis-(phosphonomethyl)amine) did not induce morphological changes in erythrocytes. The authors noted that although the effects were small, there was a stronger effect of the impurities and metabolites of glyphosate technical than of the parent compound at the higher concentrations tested.

Discussion and OCS conclusion

The OCS agreed with the conclusions reached by Kwiatkowska et al. (2014) that: glyphosate technical and the impurities and metabolites tested were not haemolytic and met-Hb producing in erythrocytes at concentrations up to 5 mM (0.85 mg/mL); the impurities and metabolites of glyphosate, particularly PMIDA, may be more haemolytic and met-Hb forming in erythrocytes than glyphosate technical; the glyphosate impurity, bis-(phosphonomethyl)amine, displays significantly more ROS producing potential than glyphosate technical and results in changes to the erythrocyte cell membrane at 5 mM (0.85 mg/mL); and no positive control agents were used for ROS investigations; hence the toxicological or biological relevance of the change in the levels of ROS production is unclear.

4.3 Conclusions and recommendations to the APVMA

The Tier 2 assessment involved:

- evaluation of 19 studies relevant to the carcinogenicity classification of glyphosate (Table 6, Appendix A)
  - 12 genotoxicity studies
  - 5 oxidative stress studies
  - 1 epidemiology study
  - 1 classification review report.

The Tier 2 assessment did not include a detailed review of the epidemiological studies or studies that evaluated the possible carcinogenicity of glyphosate-based formulations, as a number of international reviews of the IARC Monograph were undertaken concurrently with the OCS assessment. A total of 47 studies that were not reviewed by the EU RAR and 19 studies that were reviewed by the EU RAR (Table 7) were not reviewed by the OCS in the Tier 2 assessment of glyphosate because their relevance to the carcinogenicity classification for humans was unclear. The APVMA will rely on international assessments of these studies to determine whether glyphosate should be placed under formal reconsideration.

Tier 2 assessment summary

Animal carcinogenicity studies

The OCS critically evaluated one publicly available study (Greim et al. 2015) that reviewed animal carcinogenicity studies generated by industry to support registration applications (refer to Appendix C). The review paper included nine rat and five mouse studies in a weight of evidence assessment of the carcinogenicity of glyphosate that included a review of ADME, acute toxicity, genotoxicity, epidemiology and animal chronic toxicity studies.

The authors refer to an article that qualitatively analysed the outcomes from seven cohort studies and 14 case-control studies that examined an association between glyphosate and cancers. No consistent pattern of positive associations between total cancer or site-specific cancer in adults or children exposed to glyphosate was demonstrated (Mink et al. 2012). All studies cited by Mink et al. (2012) were referenced in the IARC Monograph and five (Nordstrom et al. 1998; Hardell & Eriksson 1999; McDuffie et al. 2001; Hardell et al. 2002; De Roos et al. 2005) were included in a previous assessment of glyphosate by the OCS in 2005, which concluded that glyphosate is not mutagenic or carcinogenic and it is unlikely that exposure to glyphosate is associated with an increased risk of NHL. Of the remaining studies cited by Mink et al. (2012), four (Brown et al. 1990; Cantor et al. 1992; Carreon et al. 2005; Andreotti et al. 2009) were considered during the Tier 1 assessment as not appropriate for review because glyphosate was not referred to in the abstract and the remaining 12 were identified as requiring additional assessment in order to determine their relevance to the assessment. Thus, a detailed appraisal of this paper was not conducted by the OCS as a part of the Tier 2 assessment.

Greim et al. (2015) evaluated five chronic toxicity/carcinogenicity studies (minimum 18 months) in mice. No treatment-related increases in tumour incidence were evident. Greim et al. (2015) evaluated nine unpublished chronic toxicity/carcinogenicity (24 to 29 months) studies in rats. Again, none of these studies reported treatment-related increases in tumour incidence. Greim et al. (2015) considered the results from the animal studies in conjunction with results from human carcinogenicity epidemiology conclusions reported by Mink et al. (2012)[1] and concluded that glyphosate is not carcinogenic. They noted that while some studies reported an increase in a specific neoplasm at the highest dose, the pooled data failed to identify any consistent pattern of neoplasm development or dose-response relationship. The OCS agreed with the authors' conclusion that the observed effects were not consistent or reproducible and were not treatment related and that the evidence indicates that glyphosate is not carcinogenic in animals.

Genotoxicity

The OCS appraised 11 studies and one review paper that assessed the genotoxicity of glyphosate. Of these studies, six assessed genotoxicity via the comet assay (or single cell gel electrophoresis; SCGE) *in vitro*, using lymphocytes (Mladinic et al. 2009a; Mladinic et al. 2009b; Alvarez-Moya et al. 2014), HepG2 cells (liver carcinoma cells) (Gasnier et al. 2009), Hep-2 cells (epithelial carcinoma cells derived from a cervical cancer) (Manas et al. 2009), GM38 cells (diploid fibroblast cells) or HT1080 cells (fibrocarcinoma cells) (Monroy et al. 2005). All of these studies were considered by the EFSA RAR (2015).

Of the six studies that assessed genotoxicity via the comet assay, one was considered to provide supporting information (Mladinic et al. 2009a), while four studies (Gasnier et al. 2009; Manas et al. 2009; Mladinic et al. 2009b; Alvarez-Moya et al. 2014) were not considered suitable for risk assessment purposes due to a number of reporting and methodological limitations that questioned either the interpretation or relevance of the results to humans. In one study, results from one experiment were considered unreliable while results from another experiment were considered to provide supporting evidence (Monroy et al. 2005). The relevance of a number of these studies to human exposure to glyphosate was questioned either because the exposure methods utilised are not considered relevant to humans (Alvarez-Moya et al. 2014), the observed positive results may in fact be due to

[1] Mink et al (2012) concluded that there was no consistent evidence of an association between exposure to glyphosate and cancer in humans.

cytotoxicity (Monroy et al. 2005) and/or excessively high concentrations of glyphosate that humans are highly unlikely to be exposed to were tested (Gasnier et al. 2009).

Six studies assessed genotoxicity of glyphosate using the *in vivo* micronucleus assay in various strains of mice, while one utilised the *in vitro* micronucleus assay in human lymphocytes. One *in vivo* dietary study was considered to provide supporting information only (Chan & Mahler 1992), as positive (quality) control animals were treated for only 4 weeks (compared with 13 weeks for treated animals). The suitability of the remaining five *in vivo* studies (Rank et al. 1993; Bolognesi et al. 1997; Manas et al. 2009; Mladinic et al. 2009b; Prasad et al. 2009) for risk assessment purposes was questioned due to a number of reporting and methodological limitations, particularly because they utilised the ip administration route, which is not relevant for human exposure scenarios. The proposed genotoxicity mechanism identified *in vitro* by Mladinic et al. (2009b) may not be relevant to human exposure, as the authors' claims that the concentrations of glyphosate tested correspond to acceptable safety levels based on evaluated *in vitro* endpoints have not been validated *in vivo* and positive and negative control results were virtually indistinguishable.

Three studies assessed genotoxicity via chromosomal aberration studies in mouse bone marrow cells (Li & Long 1988; Prasad et al. 2009) and human lymphocytes (Manas et al. 2009). However, two studies utilised the ip administration route (Li & Long 1988; Prasad et al. 2009) and one also utilised DMSO as a solvent (instead of water) (Prasad et al. 2009), thus the results may not be relevant to realistic human exposure scenarios. The study by Manas et al. (2009) did not report replicate data and did not concurrently assess cytotoxicity, hence it is difficult to determine whether the results are in fact due to genotoxicity or cytotoxicity.

A number of studies utilised other methods to assess genotoxicity, including applying centromere probes to analyse micronuclei and nuclear instability in human lymphocytes (Mladinic et al. 2009a), SCE in human lymphocytes and the alkaline elution assay to assess single strand DNA breaks (Bolognesi et al. 1997), microbial genotoxicity tests and *in vitro* mammalian genotoxicity tests (Li & Long 1988; Rank et al. 1993; Bolognesi et al. 1997). With the exception of the negative chromosome aberration study results (which were considered suitable as supporting information only), the negative results obtained by Li & Long (1988) were considered suitable for risk assessment purposes, while various reporting and methodological limitations questioned the suitability of the remaining studies.

Overall, the OCS concluded that the weight of evidence does not indicate that glyphosate is genotoxic in mammals at concentrations relevant to human exposure (using relevant administration routes) or below observed cytotoxicity.

Oxidative stress

Overall, seven studies assessed the potential for glyphosate to induce oxidative stress.

Three studies assessed ROS production in response to treatment with glyphosate *in vitro* in human HepG2 cells (Chaufan et al. 2014), keratinocytes (HaCaT) (Elie-Caille et al. 2010) and erythrocytes (Kwiatkowska et al. 2014). One study assessed a formulated product that is not registered for use in Australia and tested an unknown concentration of glyphosate (Chaufan et al. 2014). Another study did not test positive controls, thus the biological significance in the observed increase in ROS production is unclear (Kwiatkowska et al. 2014). Due to a number of uncertainties within the results and methodological limitations (extremely high concentrations of glyphosate and no quantitative measurement of H_2O_2) in the final study, the possibility that the observed cell responses were due to

osmotic stress rather than glyphosate toxicity could not be excluded (Elie-Caille et al. 2010). In addition to their assessment of ROS production, Chaufan et al. (2014) reported that exposure to glyphosate did not increase the activity of enzymatic (CAT, GST or SOD) or non-enzymatic (GSH) antioxidant activity in human HepG2 cells *in vitro*.

Overall, the OCS concluded that there was some limited evidence for an increase in ROS production following exposure to glyphosate, its metabolites or impurities, or a glyphosate-based formulation in *in vitro* cell culture studies using high concentrations of the test substances.

Two studies investigated caspase activity *in vivo* in male Wistar rats, following either ip administration of glyphosate (alone or in combination with other pesticides) (Astiz et al. 2009) or *in vitro* in human HepG2 cells (Chaufan et al. 2014). However, the sample size used by Astiz et al. (2009) was very small (n=4) in male rats only and ip injection is not considered relevant to human exposure. In human HepG2 cells, caspase 3/7 activity was indirectly measured in cell lysates. In addition to investigating caspase activity, Astiz et al. (2009) also investigated calpain activity *in vivo* in male Wistar rats, following exposure to glyphosate alone and in combination. However, the results reported in brain tissue are not biologically plausible for humans, due to the blood-brain barrier and rapid elimination of glyphosate via urine. Thus, the OCS concluded that there was no reliable evidence that glyphosate exposure would be likely to increase caspase or calpain activity in humans following exposure via relevant administration routes.

Bolognesi et al. (1997) investigated DNA damage in Swiss CD-1 male mice (n=3 per dose) following administration with either 300 mg/kg glyphosate technical or 900 mg/kg of Roundup® (~270 mg/kg glyphosate) via ip injection. However, no positive controls were used, so it is difficult to validate the relevance of the reported positive results. Mladinic et al. (2009a) investigated oxidative potential and impact on DNA in human lymphocytes, concluding that the lack of a clear concentration-response coupled with positive effects only being apparent at the highest concentration of glyphosate tested indicate that glyphosate does not cause oxidative DNA damage at realistic exposure concentrations.

Three studies assessed various aspects of cell morphology and structural integrity *in vitro* in human HepG2 cells (Chaufan et al. 2014), keratinocyte HaCaT cells (Elie-Caille et al. 2010) and erythrocytes (Kwiatkowska et al. 2014). It is not possible, based on the information provided in the paper by Elie-Caille et al. (2010), to determine whether glyphosate induced structural cellular changes or whether sub-confluent cells may inherently develop abnormal morphology due to the reduction in cell numbers. In human erythrocytes, glyphosate exposure did not induce morphological changes (Kwiatkowska et al. 2014). In addition, Astiz et al. (2009) investigated the integrity of the inner and outer mitochondrial membranes and peroxidation of mitochondrial membrane lipids *in vivo* in male Wistar rats (liver and brain cells). As discussed above, the results in brain tissue were not biologically plausible in humans and glyphosate alone did not significantly reduce either inner or outer mitochondrial membrane potential and did not affect mitochondrial cardiolipin content in liver (Astiz et al. 2009). The OCS concluded that there was limited evidence that a glyphosate-based formulation may be capable of stimulating apoptosis.

Using a chemometric model, one study concluded that vitamins C and E were capable of protecting cells from cytotoxic effects of glyphosate and Roundup 3 plus® (Gehin et al. 2005). However, it was difficult to determine the relevance of the small changes observed, as no positive control was employed to confirm the sensitivity of the assay and statistical analyses were not conducted. Furthermore, the authors assessed cell viability via cellular metabolic activity, but these results were not validated using specific measurements of oxidative stress, thus other

mechanisms of cytotoxicity cannot be ruled out. The relevance of these results to the *in vivo* situation are not clear, as solutions of vitamins in cell culture solutions may not accurately reflect realistic systemic human exposure.

Overall, the OCS concluded that no definitive conclusions could be drawn on the ability of glyphosate products and their associated impurities to induce oxidative stress.

Recommendations

The Tier 1 assessment identified 19 studies relevant to the carcinogenicity classification of glyphosate by IARC that had not been previously assessed by the OCS. The OCS concluded that, based on the results of the critical appraisal and the limited number of studies reviewed by the OCS, there did not appear to be any additional information to indicate that glyphosate poses a carcinogenic risk to humans, on the basis of the following:

- the weight of evidence indicates that glyphosate is not carcinogenic in laboratory animals and is not genotoxic
- the level of cytotoxicity associated with in vitro genotoxicity testing of glyphosate was significant, limiting the ability of in vitro tests to determine genotoxicity potential of glyphosate.

The OCS noted that there is some evidence that *in vitro*, glyphosate-based formulated products are more toxic to cells than glyphosate; however, this effect has not been confirmed *in vivo*. Furthermore, many of the studies exhibited significant methodological limitations, reducing the usefulness of the data.

No definitive conclusions could be drawn on the ability of glyphosate products and their associated impurities to induce oxidative stress due to the limited information available on this endpoint.

The OCS concluded that glyphosate was unlikely to pose a carcinogenicity or genotoxic risk to humans.

APPENDICES

APPENDIX A – LIST OF KEY STUDIES REFERENCED IN THE IARC MONOGRAPH 112 REVIEWED BY OCS

The studies referenced in the IARC monograph that the OCS recommended for review in their Tier 1 assessment are presented below in Table 6.

Table 6: List of studies relevant to the carcinogenicity classification of glyphosate that require evaluation

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
Alvarez-Moya, C, Silva, MR, Valdez Ramírez, CV, Gallardo, DG, Sánchez, RL, Aguirre, AC, & Velasco, AF	2014	genotoxicity	glyphosate isopropylamine	human (lymphocyte cell line)	Comparison of the in vivo and in vitro genotoxicity of glyphosate isopropylamine salt in three different organisms. Genetics and molecular biology, 37(1), 105–10	Comet assay; glyphosate isopropylamine; human lymphocytes; positive results	http://www.scielo.br/scielo.php?pid=S1415-47572014000100016&script=sci_arttext
*Astiz, M, de Alaniz, MJ & Marra, CA	2009a	oxidative stress	glyphosate	rat (unknown strain)	Effect of pesticides on cell survival in liver and brain rat tissues. Ecotoxicology and environmental safety,72(7), 2025–32	Liver and brain rat cell survival; MOA for oxidative stress seen in previous study	http://www.sciencedirect.com/science/article/pii/S0147651309001018
*Bolognesi, C, Bonatti, S, Degan, P, Gallerani, E, Peluso, M, Rabboni, R, Roggieri, P & Abbondandolo, A	1997	genotoxicity	glyphosate and Roundup	swiss CD-1 mice; human (lymphocyte cell line)	Genotoxic activity of glyphosate and its technical formulation Roundup. Journal of Agricultural and food chemistry, 45(5), 1957–62	Uses roundup and glyphosate alone; positive results seen in both	http://pubs.acs.org/doi/abs/10.1021/jf9606518
Chan, P & Mahler, J	1992	genotoxicity	glyphosate	F344/N rats and B6C3F1 mice	NTP technical report on the toxicity studies of Glyphosate (CAS No. 1071-83-6) Administered In Dosed Feed To F344/N Rats And B6C3F1 Mice. Toxicity report series, 16, 1–D3	Effects in rats and mice; no mutagenicity in salmonella; negative for LLNA	http://europepmc.org/abstract/med/12209170

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
*Chaufan, G, Coalova, I & Rios de Molina Mdel, C	2014	oxidative stress	glyphosate, AMPA and glyphosate formulation	human (HepG2 cell line)	Glyphosate Commercial Formulation Causes Cytotoxicity, Oxidative Effects, and Apoptosis on Human Cells Differences With its Active Ingredient. International journal of toxicology, 33(1), 29–38	Shows formulation increases ROS and has toxic effects not seen in glyphosate alone	http://ijt.sagepub.com/content/33/1/29.short
*Elie-Caille, C, Heu, C, Guyon, C & Nicod, L	2010	oxidative stress	glyphosate	human keratinocyte (HaCaT cell line)	Morphological damages of a glyphosate-treated human keratinocyte cell line revealed by a micro-to nanoscale microscopic investigation. Cell biology and toxicology, 26(4), 331–39	Shows the timeline of membrane damage and ROS production in human keratinocytes	http://www.ncbi.nlm.nih.gov/pubmed/20043237
*Gasnier, C, Dumont, C, Benachour, N, Clair, E, Chagnon, MC & Seralini, GE	2009	genotoxicity	glyphosate and glyphosate formulations	human (HepG2 cell line)	Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology, 262(3), 184–91	Shows effects are dependent on formulation not glyphosate concentration	http://www.sciencedirect.com/science/article/pii/S030048X09003047
*Gehin, A, Guillaume, YC, Millet, J, Guyon, C & Nicod, L	2005	oxidative stress	glyphosate and round-up	human keratinocyte (HaCaT cell line)	Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach. International journal of pharmaceutics,288(2), 219–26	Shows effects are due to formulation; uses human keratinocyte cell line	http://www.sciencedirect.com/science/article/pii/S0378517304005733

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
Greim, H, Saltmiras, D, Mostert, V & Strupp, C	2015	carcinogenicity/ epidemiology	glyphosate and glyphosate formulations	human, rat, mouse	Evaluation of carcinogenic potential of the herbicide glyphosate, drawing on tumor incidence data from fourteen chronic/carcinogenicity rodent studies. Critical reviews in toxicology, 45(3), 185–208	Shows no carcinogenic effect	http://www.tandfonline.com/doi/abs/10.3109/10408444.2014.1003423#.Vf9hMvk0VcY
JMPR	2006	classification					http://apps.who.int/iris/bitstream/10665/43624/1/9241665203_eng.pdf?ua=1
*Kier, LD & Kirkland, DJ	2013	genotoxicity	glyphosate and glyphosate formulations	in vitro and in vivo	Review of genotoxicity studies of glyphosate and glyphosate-based formulations. Critical reviews in toxicology,43(4), 283–315	Review of genotoxicity tesing for glyphosate and formulations	http://www.ncbi.nlm.nih.gov/pubmed/23480780
*Kwiatkowska, M, Huras, B & Bukowska, B	2014	oxidative stress	glyphosate, glyphosate metabolites and glyphosate impurities	human (erythrocyte cell line)	The effect of metabolites and impurities of glyphosate on human erythrocytes (in vitro). Pesticide biochemistry and physiology, 109, 34–43	Uses human erythrocytes; shows that ROS and damage only occurs at levels seen in acute poisoning	http://www.sciencedirect.com/science/article/pii/S0048357514000200
*Li, AP & Long, TJ	1998	genotoxicity	glyphosate	in vitro and in vivo	An evaluation of the genotoxic potential of glyphosate. Toxicological Sciences, 10(3), 537–46	Multiple genotoxicity tests; shows no genotoxic potential	http://toxsci.oxfordjournals.org/content/10/3/537.short

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
*Manas, F, Peralta, L, Raviolo, J, Ovando, HG, Weyers, A, Ugnia, L, Cid, MG, Larripa, I & Gorla, N	2009a	genotoxicity	glyphosate	human (Hep-2 cell line); mouse micronucleus	Genotoxicity of glyphosate assessed by the comet assay and cytogenetic tests. Environmental Toxicology and Pharmacology, 28(1), 37–41	Shows positive genotoxicity results in Hep-2 cells and micronucleus mouse test at 400 mg/kg	http://www.sciencedirect.com/science/article/pii/S1382668909000258
*Mladinic, M, Berend, S, Vrdoljak, AL, Kopjar, N, Radic, B & Zeljezic, D	2009a	genotoxicity	glyphosate	human (lymphocyte cell line)	Evaluation of genome damage and its relation to oxidative stress induced by glyphosate in human lymphocytes in vitro. Environmental and molecular mutagenesis, 50(9), 800–7	Shows no clear dose dependent effect	http://onlinelibrary.wiley.com/doi/10.1002/em.20495/abstract
*Mladinic, M, Perkovic, P & Zeljezic, D	2009b	genotoxicity	glyphosate	human (lymphocyte cell line)	Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay. Toxicology letters, 189(2), 130–7	Cytome FISH assay; shows no hazardous effect on DNA at low concentrations	http://www.sciencedirect.com/science/article/pii/S0378427409002616
*Monroy, CM, Cortes, AC, Sicard, DM & de Restrepo, HG	2005	genotoxicity	glyphosate	human (GM38 and fibrosarcoma HT1080 cell lines)	Cytotoxicity and genotoxicity of human cells exposed in vitro to glyphosate.Biomedica, 25(3), 335–45	Suggests MOA not limited to plants	http://www.scielo.org.co/scielo.php?pid=S0120-41572005000300009&script=sci_arttext&tlng=pt
Prasad, S, Srivastava, S, Singh, M & Shukla, Y	2009	genotoxicity	glyphosate	swiss albino mice	Clastogenic effects of glyphosate in bone marrow cells of Swiss albino mice. Journal of toxicology, 2009	Shows positive clastogenic and cytotoxic effects in mouse bone marrow	http://www.hindawi.com/journals/jt/2009/308985/abs/

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
*Rank, J, Jensen, AG, Skov, B, Pedersen, LH & Jensen, K	1993	genotoxicity	glyphosate isopropylamine salt and Roundup	in vitro and in vivo	Genotoxicity testing of the herbicide Roundup and its active ingredient glyphosate isopropylamine using the mouse bone marrow micronucleus test, Salmonella mutagenicity test, and Allium anaphase-telophase test. Mutation Research/Genetic Toxicology, 300(1), 29–36	Shows negative effects for glyphosate in three genotoxicity tests	http://www.sciencedirect.com/science/article/pii/0165121893901362

*Considered by EFSA (2015)

APPENDIX B – LIST OF KEY STUDIES REFERENCED IN THE IARC MONOGRAPH 112

The studies that were referenced in the IARC monograph that the OCS Tier 1 assessment concluded required further assessment to determine their relevance to the carcinogenicity classification of glyphosate are presented below in Table 7. The APVMA will rely on international assessments of these studies to determine whether glyphosate should be placed under formal reconsideration.

Table 7: List of studies recommended by the OCS for further assessment to determine if relevant to carcinogenicity classification of glyphosate

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
*Alavanja, MC, Samanic, C, Dosemeci, M, Lubin, J, Tarone, R, Lynch, CF, Knott, C, Thomas, K, Hoppin, JA, Barker, J, Coble, J, Sandler, DP & Blair, A.	2003	Carcinogenicity/ epidemiology	unknown formulation	human	Use of agricultural pesticides and prostate cancer risk in the Agricultural Health Study cohort. American Journal of Epidemiology,157(9), 800–14	No direct reference to glyphosate in abstract, increased risk to 'other pesticides' only seen in subjects with a FHx of prostate cancer	http://aje.oxfordjournals.org/content/157/9/800.short
*Astiz, M, de Alaniz, MJ, & Marra, CA.	2009b	oxidative stress	glyphosate	rat	Antioxidant defense system in rats simultaneously intoxicated with agrochemicals. Environmental toxicology and pharmacology, 28(3), 465–73	Glyphosate administered alone and in combo with other a.i.'s; unclear if results are for combo; in vivo rat model	http://www.sciencedirect.com/science/article/pii/S1382668909001392
Astiz, M, Hurtado de Catalfo, GE., García, MN, Galletti, SM, Errecalde, AL, de Alaniz, MJ, & Marra, CA.	2013	oxidative stress	glyphosate	wistar rat	Pesticide-induced decrease in rat testicular steroidogenesis is differentially prevented by lipoate and tocopherol. Ecotoxicology and environmental safety, 91, 129–38	Oxidative stress seen in testicular cells; investigates antioxidant treatment after administration; unclear if administered in combo	http://www.sciencedirect.com/science/article/pii/S0147651313000389

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
Benachour, N, & Séralini, GE.	2009	MOA	Roundup	human (umbilical, embryonic, placental cell lines)	Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells.Chemical research in toxicology, 22(1), 97–105	Uses glyphosate formulations, investigates metabolites	http://pubs.acs.org/doi/abs/10.1021/tx800218n
Benachour, N, Sipahutar, H, Moslemi, S, Gasnier, C, Travert, C, & Séralini, GE.	2007	MOA	Roundup (bioforce)	human (embryonic and placental cell lines)	Time-and concentration-dependent effects of roundup on human embryonic and placental cells. Archives of Environmental Contamination and Toxicology,53(1), 126–33	Uses glyphosate formulations, investigates toxicity and endocrine-disruption	http://link.springer.com/article/10.1007/s00244-006-0154-8
*Bolognesi, C, Carrasquilla, G, Volpi, S, Solomon, KR, & Marshall, EJP.	2009	genotoxicity/epidemiology	glyphosate + cosmo-flux	human	Biomonitoring of genotoxic risk in agricultural workers from five Colombian regions: association to occupational exposure to glyphosate.Journal of Toxicology and Environmental Health, Part A, 72(15-16), 986–97	Columbian aerial spray program; uses formulation as exposure to glyphosate; measurement of binucleated lymphocytes with micronuclei as DNA damage	http://www.tandfonline.com/doi/abs/10.1080/15287390902929741#.Ve0iNfk0VcY
Brewster, DW, Warren, J, & Hopkjns, WE.	1991	metabolism	glyphosate	SD rat	Metabolism of glyphosate in Sprague-Dawley rats: tissue distribution, identification, and quantitation of glyphosate-derived materials following a single oral dose.Toxicological Sciences, 17(1), 43–51	Tissue distribution study, shows no persistence in body after single oral dose	http://toxsci.oxfordjournals.org/content/17/1/43.short

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
Brown, LM, Burmeister, LF, Everett, GD, & Blair, A.	1993	carcinogenicity/epidemiology	unknown formulation	human	Pesticide exposures and multiple myeloma in Iowa men. Cancer Causes & Control, 4(2), 153–6	No direct reference to glyphosate or roundup; shows little evidence of association between pesticides and multiple myeloma	http://link.springer.com/article/10.1007/BF00053156
Cattani, D, Cavalli, VLDLO, Rieg, CEH, Domingues, JT, Dal-Cim, T, Tasca, CI, & Zamoner, A.	2014	oxidative stress	Roundup	rat	Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity. Toxicology, 320, 34–45	Uses formulation; neurotoxic effects on rat hippocampus	http://www.sciencedirect.com/science/article/pii/S0300483X14000493
Çavuşoğlu, K, Yapar, K, Oruç, E, & Yalçın, E.	2011	oxidative stress	Roundup	SA mouse	Protective effect of Ginkgo biloba L. leaf extract against glyphosate toxicity in Swiss albino mice.Journal of medicinal food, 14(10), 1263–72	Uses formulation; i.p. to mice; studies the effect of Ginkgo against effects seen	http://online.liebertpub.com/doi/abs/10.1089/jmf.2010.0202
Chruscielska, K, Brzezinski, J, Kita, K, Kalhorn, D, Kita, I, Graffstein, B, & Korzeniowski, P.	2000	toxicity			Glyphosate. Evaluation of chronic activity and possible far-reaching effects. Part 1. Studies on chronic toxicity. Pestycydy, 3	Chronic toxicity study review	
Coalova, I, de Molina, MDCR, & Chaufan, G.	2014	oxidative stress	atanor + impacto (adjuvant)	human (Hep-2 cell line)	Influence of the spray adjuvant on the toxicity effects of a glyphosate formulation. Toxicology in Vitro,28(7), 1306–11	Uses formulation and adjuvant on Hep-2 cell line; shows toxicity and ROS	http://www.sciencedirect.com/science/article/pii/S0887233314001295

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
Cocco, P, Satta, G, Dubois, S, Pili, C, Pilleri, M, Zucca, M, 't Mannetje AM, Becker, N, Benavente, Y, de Sanjose, S, Foretova, L, Staines, A, Maynadie, M, Nieters, A, Brennan, P, Miligi L, Enna, MG & Boffetta, P.	2012	carcinogenicity/epidemiology	unknown formulation	human	Lymphoma risk and occupational exposure to pesticides: results of the Epilymph study. Occupational and environmental medicine, oemed-2012	No direct reference to glyphosate; based on pesticide exposure determined via survey	http://oem.bmj.com/content/early/2012/10/31/oemed-2012-100845.short
Culbreth, ME, Harrill, JA, Freudenrich, TM, Mundy, WR, & Shafer, TJ.	2012	MOA	glyphosate	human; mouse	Comparison of chemical-induced changes in proliferation and apoptosis in human and mouse neuroprogenitor cells. Neurotoxicology, 33(6), 1499–510	Apoptosis induced by glyphosate, neurodevelopmental study; uses human and mouse neural cells	http://www.sciencedirect.com/science/article/pii/S0161813X12001271
Dennis, LK, Lynch, CF, Sandler, DP, & Alavanja, MC.	2010	carcinogenicity/epidemiology	unknown formulation	human	Pesticide use and cutaneous melanoma in pesticide applicators in the agricultural heath study. Environmental Health Perspectives, 118(6), 812–7	Uses formulation; no results relating to glyphosate	http://www.ladep.es/ficheros/documentos/10(35).pdf
*De Roos, A, Zahm, SH, Cantor, KP, Weisenburger, DD, Holmes, FF, Burmeister, LF, & Blair, A.	2003	carcinogenicity/epidemiology	unknown formulation	human	Integrative assessment of multiple pesticides as risk factors for non-Hodgkin's lymphoma among men.Occupational and Environmental Medicine, 60(9), e11–e11	Uses formulation; shows positive trend with NHL	http://oem.bmj.com/content/60/9/e11.short

Author(s)	Year	Endpoint	Formulation type (if stated)	Species	Title/publication details	Comments	Website
*Dimitrov, BD, Gadeva, PG, Benova, DK, & Bineva, MV.	2006	genotoxicity	Roundup	mouse (bone marrow)	Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems. Mutagenesis, 21(6), 375–82	Comparative study using glyphosate formulation; negative results	http://mutage.oxfordjournals.org/content/21/6/375.short
*Engel, LS, Hill, DA, Hoppin, JA, Lubin, JH, Lynch, CF, Pierce, J, Samanic, C, Sandler, DP, Blair, A & Alavanja, MC.	2005	carcinogenicity/epidemiology	unknown formulation	human	Pesticide use and breast cancer risk among farmers' wives in the agricultural health study. American Journal of Epidemiology,161(2), 121–35	Uses formulation; glyphosate not directly referenced in the abstract; no clear association with breast cancer	http://aje.oxfordjournals.org/content/161/2/121.short
*Eriksson, M, Hardell, L, Carlberg, M, & Åkerman, M.	2008	carcinogenicity/epidemiology	unknown formulation	human	Pesticide exposure as risk factor for non-Hodgkin lymphoma including histopathological subgroup analysis. International Journal of Cancer, 123(7), 1657–63	Uses formulation; results were not adjusted for multiple exposures; shows increased risk of NHL for glyphosate	http://onlinelibrary.wiley.com/doi/10.1002/ijc.23589/pdf

*Considered by EFSA (2015)

APPENDIX C – ASSESSMENT OF THE REVIEW PAPER BY GREIM ET AL 2015

Greim, H, Saltmiras, D, Mostert, V & Strupp, C 2015, 'Evaluation of carcinogenic potential of the herbicide glyphosate, drawing on tumor incidence data from fourteen chronic/carcinogenicity rodent studies', Crit Rev Toxicol, vol. 45, pp. 185–208.

Greim et al. (2015) reviewed animal carcinogenicity studies generated by industry for regulatory bodies (nine rat and five mouse studies) to determine a weight-of-evidence carcinogenicity classification for glyphosate. The weight-of-evidence approach included a review of ADME, acute toxicity, genotoxicity, epidemiology and animal chronic toxicity studies.

Epidemiology

Greim et al. (2015) refer to an article that qualitatively analysed the outcomes from seven cohort studies and 14 case-control studies that examined an association between glyphosate and cancers (Mink et al. 2012). No consistent pattern of positive associations between total cancer or site-specific cancer in adults or children exposed to glyphosate was demonstrated (Mink et al. 2012). This paper was not critically appraised as part of the Tier 2 assessment. All studies cited by Mink et al. (2012) were referenced in the IARC Monograph and five have been previously reviewed by the OCS.

Chronic toxicity studies in animals

Several one year chronic toxicity studies in animals were reviewed but, as they were not designed to detect neoplasms, less weight was placed on the results obtained from these studies than those obtained from studies of longer duration. In five one-year repeat-dose studies using dogs (beagles), no pre-neoplastic lesions were observed by histopathological observations. The lowest relevant No Observed Adverse Effect Level (NOAEL; highest NOAEL below the lowest LOAEL) reported was 500 mg/kg bw/d, based on minor effects on body weight gain and soft stools, indicating low toxicity following repeat exposures in dogs; however, the maximum tolerated dose (MTD) was not reached in that study. Current internationally-recognised test guidelines (IUPAC 1997) stipulate that the highest dose tested should be at the MTD, interpreted as the dose causing non-lethal toxicity (usually reduced body weight gain of 10% or more).

In rats, no pre-neoplastic lesions were noted when up to 20 000 ppm glyphosate technical was provided in the diet for one year. Although an increased incidence of mild focal basophilia and hypertrophy of the acinar cells of the parotid salivary gland at the highest dose was considered to be treatment related, it was attributed to oral irritation from ingestion of glyphosate, rather than toxicity. This was confirmed by mode of action studies with citric acid, which is a non-toxic organic acid with similar irritation properties to glyphosate. The reported NOAEL for this study was 560 or 671 mg/kg bw/d in males and females, respectively (8000 ppm), based on reduced body weight; however, there were indications that the decrease in body weight may in fact reflect reduced food consumption as a result of reduced palatability of the diets containing higher concentrations of glyphosate. Similarly to the studies conducted in dogs, the results of this chronic toxicity study conducted in rats indicate low toxicity of glyphosate technical following long-term daily exposure.

Carcinogenicity studies: mice

Greim et al. (2015) evaluated five chronic toxicity/carcinogenicity studies (minimum 18 months) in mice (Table 8). Four of these studies were considered reliable and were performed according to GLP following OECD TGs.

Table 8: Summary of carcinogenicity studies in mice (referenced in Greim et al. (2015))

Strain	Dose	Carcinogenicity findings	Discussion and conclusion
Study 10: (Knezevich & Hogan 1983)			
CD-1 50/sex/dose and 10/sex/dose (haematology at 12 and 18 months) and 12M/dose + all surviving F (haematology at termination)	1000, 5000, 30 000 ppm in the diet (157/190, 814/955, 4841/5874 mg/kg bw/d M/F) for 24 months	Spontaneous tumours noted at all doses	Non-GLP study (conducted prior to introduction of GLP), but highly compliant with OECD TG 451, which was adopted while the study was ongoing (1981) No tumours or lesions reflected a dose–response pattern; therefore not considered treatment related **Not carcinogenic**
Study 11: (Atkinson et al. 1993a)			
CD-1 50/sex/dose	0, 100, 300, 1000 mg/kg bw/d in the diet for 24 months	Spontaneous tumours noted at all doses No statistically significant increases in tumour incidence	Conducted in accordance to GLP and OECD guidelines No tumours or lesions exhibited significance or dose–response, therefore were not considered treatment related Tumour incidence was within the range of historical control data **Not carcinogenic**
Study 12: (Sugimoto 1997)			
ICR-CD-1 50/sex/dose	1600, 8000, 40 000 ppm in the diet (165/153, 838/787, 838/787, 4348/4116 mg/kg bw/d M/F) for 18 months	Spontaneous tumours noted at all doses No statistically significant increases in tumour incidence	Conducted in accordance to GLP and OECD guidelines No tumours or lesions exhibited significance or dose–response, therefore were not considered treatment related **Not carcinogenic**
Study 13: (Kumar 2001)			
Swiss albino 50/sex/dose	0, 100, 1000, 10 000 ppm in the diet (0, 14.5/15.0, 150/151, 1454/1467 mg/kg bw/d M/F) for 18 months	Statistically significant increased incidence of malignant lymphoma (HD only)	Conducted in accordance to GLP and OECD guidelines but possible viral infection within the colony may have confounded the interpretation of results Increased incidence of malignant lymphoma in the HD group could not be excluded as being treatment related as the increase was significant in males and outside of the historical control range. However, results are inconsistent with other studies at similar

Strain	Dose	Carcinogenicity findings	Discussion and conclusion
			doses and this tumour is a common spontaneous tumour in this strain of mouse No other tumours or lesions exhibited dose-response; therefore were not considered treatment related **Limited evidence of carcinogenicity**
Study 14: (Wood et al. 2009a)			
CD-1 51/sex/dose	0, 500, 1500, 5000 ppm in the diet (0, 85, 267, 946 mg/kg bw/d) for 18 months	Spontaneous tumours noted in males Dose-response seen in incidence of bronchio-alveolar adenocarcinoma and malignant lymphoma in males only	Conducted in accordance to GLP and OECD guidelines. MTD not reached. Bronchio-alveolar adenocarcinoma and malignant lymphoma are common in aging mice were only noted in one sex and were only significant at the HD, therefore it was not considered to be treatment related. **Not carcinogenic**

F: Females; M: Males; HD: high dose; MTD: maximum tolerated dose; GLP: good laboratory practice; OECD (TG): the organisation for economic co-operation and development (testing guideline)

Table 9 below summarises the neoplasms evaluated by the studies presented in Greim et al. (2015) and the overall conclusion regarding carcinogenicity at the doses of glyphosate administered to either rats or mice.

Table 9: Summary of neoplasms evaluated by Greim et al. (2015) and overall carcinogenicity conclusion

Species (sex)	Neoplasms evaluated	Greim et al. (2015) conclusion
Rat (M)	Pancreatic islet cell adenoma Pituitary adenoma Pituitary carcinoma Testes interstitial cell (Leydig) Thyroid C cell adenoma Hepatocellular adenoma Hepatocellular carcinoma Benign keratoacanthoma (skin) Lung adenoma	No dose response (3–1290 mg/kg bw/d for up to 24 months)
Rat (F)	Pancreatic islet cell adenoma Pituitary adenoma Pituitary carcinoma Thyroid C cell adenoma Hepatocellular adenoma Hepatocellular carcinoma Mammary gland fibroadenoma Mammary gland adenocarcinoma	No dose response (3–1740 mg/kg bw/d for up to 24 months)

Species (sex)	Neoplasms evaluated	Greim et al. (2015) conclusion
Mouse (M, F)	Bronchiolar-alveolar adenoma Bronchiolar-alveolar adenocarcinoma Bronchiolar-alveolar carcinoma Hepatocellular adenoma Hepatocellular carcinoma Malignant lymphoma Myeloid leukemia Pituitary adenoma	No dose response (14.5–5874 mg/kg bw/d for up to 24 months)

F: Females; M: Males

Greim et al. (2015) Mink et al. (2012)concluded that, based on the animal data and epidemiological data reviewed, glyphosate is not carcinogenic. They noted that some studies reported an increase in a specific neoplasm at the high dose but that the pooled data failed to identify any consistent pattern of neoplasm development or dose-response. Thus, the authors concluded that the observed effects were not consistent or reproducible and were not treatment related.

Carcinogenicity studies: rats

Greim et al. (2015) evaluated nine chronic toxicity/carcinogenicity (24 to 29 months) studies in rats submitted by industry (Table 10). Seven of these studies were conducted under Good Laboratory Practice (GLP), while two were not. Of these two non-GLP studies, one was conducted prior to the introduction of GLP.

Table 10: Summary of carcinogenicity findings in rats referenced in Greim et al. (2015)

Strain, sample size	Dose	Carcinogenicity findings	Discussion and conclusion
Study 1: (Lankas 1981)			
SD 50/sex/dose	0, 30, 100, 300 ppm in the diet (0, 3.05/3.37, 10.3/11.22, 31.49/34.04 mg/kg bw/d M/F) for up to at least 26 months	Spontaneous neoplasms noted Increased incidence in mammary and pituitary adenomas with age	Non-GLP study (conducted prior to introduction of GLP), but highly compliant with OECD TG 453 (1981) MTD not reached Increased incidence in mammary and pituitary adenomas with age not considered treatment related Increased incidence of interstitial cell tumours in testes within normal biological variation for site and strain Dose-response absent **Not carcinogenic** Due to reporting and methodology deficiencies (based on contemporary standards) this study was not considered reliable (ie supportive only)

Strain, sample size	Dose	Carcinogenicity findings	Discussion and conclusion
Study 2: (Stout & Ruecker 1990)			
SD 50/sex/dose 10/sex/dose (interim sacrifice at 12 months)	0, 2000, 8000, 20 000 ppm in the diet (0, 89/113, 362/457, 940/ 1183 mg/kg bw/d M/F) for 24 months	Statistically significant increase in benign tumours in the thyroid (C-cell adenoma) in males receiving 8000 ppm sacrificed after 12 months only Significantly increased incidence in benign pancreatic islet cell adenoma in males receiving 2000 or 20 000 ppm only	Conducted in accordance with contemporary version of OECD TG 453 (1990) MTD achieved in highest dose group Benign tumours did not exhibit dose-response and did not progress to malignant neoplasms, therefore were not considered treatment related **Not carcinogenic**
Study 3: (Atkinson et al. 1993b)			
SD 50/sex/dose 35/sex/dose (interim sacrifice at 12 months)	0, 10, 100, 300, 1000 mg/kg bw/d in the diet for 24 months.	Neoplasms occurred with similar frequency in control and test groups.	Conducted in accordance with GP and OECD guidelines. MTD achieved in highest dose group. Dose-responses not evident. No statistically significant increases vs controls were noted for any tumour type, therefore not considered treatment related. **Not carcinogenic.**
Study 4: (Suresh 1996)			
Wistar 50/sex/dose 10 control and 20/sex/ high dose (interim sacrifice at 12 months)	0, 100, 1000, 10 000 ppm in the diet (0, 7.4, 73.9, 740.6 mg/kg bw/d) for 24 months	Spontaneous benign tumours noted at all dose levels Hepatocellular adenomas and carcinomas occurred more frequently with age	Conducted in accordance to GLP and OECD guidelines Benign tumours and liver tumours (benign and malignant) did not exhibit a dose-response, therefore were not considered treatment related MTD may not have been reached **Not carcinogenic**
Study 5: (Excel 1997)			
SD 50/sex/dose and 20/sex/dose (interim sacrifice at 12 months)	0, 3000, 15 000, 25 000 ppm in the diet (0, 150/210, 780/1060, 1290/1740 mg/kg bw/d M/F) for 24 months	No significant increase in tumour incidence	Lower than expected background tumour incidence. Test substance was not adequately characterised. Methodology deviations from OECD TG 453 **Not carcinogenic** Due to reporting and methodology deficiencies this study was not considered to be reliable (ie supportive only)

Strain, sample size	Dose	Carcinogenicity findings	Discussion and conclusion
Study 6: (Enomoto 1997)			
SD 50/sex/dose and 30/sex/dose (interim sacrifices at 26, 52 and 78 weeks)	0, 3000, 10 000, 30 000 ppm in the diet (0, 104/115, 354/393, 1127/1247 mg/kg bw/d M/F) for 24 months	No significant increase in incidence of pituitary adenoma (HD M), skin keratoacanthoma (HD M), mammary gland fibroadenomas (LD & MD F) Increased incidence in mammary and pituitary adenomas with age	Conducted in accordance with GLP and OECD guidelines Increased incidence in mammary and pituitary adenomas with age not considered treatment related Skin keratoacanthoma in aging male mice is common and not considered treatment related MTD achieved in highest dose group (decreased body weight, increased caecum weight, distension of caecum, loose stools, skin lesions) **Not carcinogenic**
Study 7: (Brammer 2001)			
Wistar 52/sex/dose and 12/sex/dose (interim sacrifice at 12 months)	0, 2000, 6000, 20 000 ppm via the diet (0, 121/145, 361, 439, 1214/ 1498 mg/kg bw/d M/F) for 12 months.	Significant trend for increased hepatocellular adenoma at 20 000 ppm in males only	Conducted in accordance with GLP and OECD guidelines Increased incidence in hepatocellular adenoma did not exhibit dose–response and did not exhibit pre-neoplastic foci or progression; therefore was not considered treatment related **Not carcinogenic**
Study 8: (Wood et al. 2009b)			
Wistar 51/sex/dose	0, 1500, 5000, 15 000 (progressed to 24 000) ppm in the diet (0, 86/105, 285/349, 1077/1382 mg/kg bw/d M/F) for 24 months	Increased incidence of benign skin keratoacanthoma (M) and malignant mammary gland tumours (F)	Conducted in accordance to GLP and OECD guidelines Skin keratoacanthoma and mammary gland tumours did not exhibit dose-response, therefore were not considered treatment related Mammary gland tumours are a common age-related tumour in Wistar rats **Not carcinogenic**
Study 9: (Chruscielska et al. 2000)			
Wistar-RIZ 85/sex/dose and 10/sex/dose (interim sacrifice at 6, 12 and 18 months)	0, 300, 900, 2700 mg/L in drinking water (0, 1.9/2.2, 5.7/6.5, 17/19 mg/kg bw/d M/F) for 24 months	No pre-neoplastic lesions or neoplasms identified	Methodology deviations from OECD TG 453 Water consumption data not reported, therefore daily doses were estimated Glyphosate-based formulation (13.85%) MTD not reached **Not carcinogenic** Due to reporting and methodology deficiencies this study was not considered to be reliable (ie supportive only)

SD: Sprague Dawley; MTD: maximum tolerated dose; HD: high dose; MD: median dose; LD: low dose; M: males; F: females; GLP: good laboratory practice; OECD (TG): the organisation for economic co-operation and development (testing guideline)

Discussion and OCS conclusion

All of the studies evaluated by Greim et al. (2015) were assessed and cited by the EFSA RAR (2015). Based on a review of the supplementary raw data provided within the documents and in parallel with the conclusions of the EFSA RAR (2015), the OCS conclusions are consistent with Greim et al. (2015) and the EFSA RAR (2015) that the evidence indicates that glyphosate is not carcinogenic in animals.

As part of the review of Greim et al. (2015), the cited review paper by Mink et al. (2012) was considered. All of the studies reviewed by Mink et al. (2012) were referenced by the IARC monograph. Five of the studies cited by Mink et al. (2012) were reviewed previously by the OCS in 2005 (Nordstrom et al. 1998; Hardell & Eriksson 1999; McDuffie et al. 2001; Hardell et al. 2002; De Roos et al. 2005). The OCS weight-of-evidence conclusion in 2005 was that glyphosate is not mutagenic or carcinogenic and it is unlikely that exposure to glyphosate is associated with an increased risk of NHL. Of the remaining studies cited by Mink et al. (2012), four (Brown et al. 1990; Cantor et al. 1992; Carreon et al. 2005; Andreotti et al. 2009) were considered during the Tier 1 assessment as not appropriate for review due to a lack of reference to glyphosate in the abstract and the remaining 12 were indicated in the proposed Tier 2, Part 2 review of the monograph. However, as these papers were critically evaluated by the international JMPR assessment of glyphosate, the proposed Tier 2, Part 2 assessment will not be undertaken by the OCS.

ABBREVIATIONS

ADME	Absorption, distribution, metabolism and excretion
AMPA	Aminomethylphosphonic acid
ANOVA	Analysis of variance
APVMA	Australian Pesticides and Veterinary Medicines Authority
^{14}C	Carbon-14
CAS No.	Chemical abstracts service number
CAT	Catalase
CHO	Chinese hamster ovary
CI	Confidence interval
Cyt_c	cytochrome *c*
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
F	female
F1	First filial generation
F3	Third filial generation
FAO	Food and Drug Administration of the United Nations
FRAP	Ferric-inducing ability of plasma
FSC-A	Forward scatter pulse area
GLP	Good Laboratory Practice
GSH	Glutathione
GST	Glutathione-S-transferase
HD	High dose
H_2DCF	2′,7′-dichlorodihydrofluorescein
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
HGPRT	hypoxanthine-guanine phosphoribosyl transferase

HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
IARC	International Agency for Research on Cancer
IC_{50}	half maximal inhibitory concentration
IC_{65}	65% maximal inhibitory concentration
ip	intraperitoneal
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
LC_{20}	Lethal concentration causing 20% mortality
LC_{50}	Lethal concentration causing 50% mortality
LD	Low dose
LD_{50}	Lethal dose
M	male
MD	Median dose
met-Hb	methaemoglobin
mg/kg bw	milligrams per kilogram of bodyweight
mg/kg bw/day	milligrams per kilogram of bodyweight per day
mg/mL	milligrams per mil
MI	Mitotic index
µM	micromolar
µm	micrometre
mM	millimolar
MNE	Micronucleated erythrocytes
MNPCE	Micronucleated polychromatic erythrocyte
MNT	Micronucleus test
MTD	Maximum tolerated dose
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	*N*-acetylcysteine
NCE	normochromatic erythrocyte

NDEA	N-Nitrosodiethylamine
NHL	non-Hodgkin's lymphoma
nm	nanometre
NOAEL	No observed adverse effect level
OCS	Office of Chemical Safety
OECD	The Organisation for Economic Co-operation and Development
OECD TG	OECD Testing Guidelines
OR	Odds ratio
PBL	Peripheral blood lymphocyte
PCE	Polychromatic erythrocyte
pH	Potential hydrogen
PMIDA	N-(phosphonomethyl)iminodiacetic acid
ppm	parts per million
RAR	Renewal assessment rapport
ROS	Reactive oxygen species
S9	Aroclor 1254-induced rat liver homogenate supernatant
SCE	Sister chromatid exchange
SCGE	Single-cell gel electrophoresis (comet assay)
SD	Sprague Dawley (rat)
SOD	Superoxide dismutase
SSC-A	Side scatter pulse area
TBARS	Thiobabituric acid reactive substances
WHO	World Health Organization
w/v	weight per volume
w/w	weight per weight
8-OHdG	8-hydroxy-2'-deoxyguanosine

GLOSSARY

First filial generation	The offspring of a genetically specified mating
Inhibitory concentration	The concentration of a substance required to inhibit a biological or biochemical function by half
Lambda	Wavelength
Lethal concentration	The concentration of a substance required to kill 20% (LC_{20}) or 50% (LC_{50}) of the population
No observed adverse effect level	Greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or lifespan of the target organism under defined conditions of exposure
Odds ratio	A measure of association between an exposure and an outcome. The odds ratio 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
p-value	The level of marginal significance within a statistical hypothesis test. Represents the probability of the occurrence of an event and is used as an alternative to rejection points to provide the smallest level of significance at which the null hypothesis would be rejected
Third filial generation	The offspring of two first filial generation individuals
weight per volume	A measure of the concentration of a solution used where a solid chemical is dissolved in a liquid
weight per weight	A measure of the concentration of a solution used where the weight of each chemical is used and not the volume
95% Confidence Interval (CI)	A CI measures the probability that a population parameter will fall between the two set values. For a 95% CI, there is a 95% chance that the parameter will fall between the two values

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