Title: **Review of Genotoxicity Studies of Glyphosate and Glyphosate-Based Formulations**

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Abbreviations: a.e., acid equivalents; a.i., active ingredient; CB MN, cytokinesis block micronucleus; GBF, glyphosate-based formulation; i.p., intraperitoneal; MN, micronucleus; MN PCE, micronucleated polychromatic erythrocyte; NCE, normochromatic erythrocyte; PCE, polychromatic erythrocyte; p.o., oral administration; SCE, sister chromatid exchange; SCGE, single cell gel electrophoresis (Comet assay); OECD, Organization for Economic Co-operation and Development; S9, 9000xg liver homogenate supernatant; 8-OHdG , 8-hydroxydeoxyguanosine.

Abstract:

An earlier review of the toxicity of glyphosate and the original Roundup™-branded formulation concluded that neither glyphosate nor the formulation poses a risk for the production of heritable/somatic mutations in humans (Williams et al., 2000). The present review of subsequent genotoxicity publications and regulatory studies of glyphosate and glyphosate-based formulations (GBF's) incorporates all of the findings into a weight of evidence for genotoxicity. An overwhelming preponderance of negative results in well-conducted bacterial reversion and *in vivo* mammalian micronucleus and chromosome aberration assays indicates that glyphosate and typical GBF's are not genotoxic in these core assays. Negative results for *in vitro* gene mutation and a majority of negative results for chromosome effects assays in mammalian cells add to the weight of evidence that glyphosate is not typically genotoxic for these endpoints in mammalian systems. Mixed results were observed for micronucleus assays of GBF's in non-mammalian systems. Reports of positive results for DNA damage endpoints indicate that glyphosate and GBF's tend to elicit DNA damage effects at high or toxic dose levels, but the data suggest that this is due to cytotoxicity rather than DNA interaction with GBF activity perhaps associated with the surfactants present in many GBF's. Glyphosate and typical GBF's do not appear to present significant genotoxic risk under normal conditions of human or environmental exposures.

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1. Introduction

Glyphosate is the active ingredient (a.i.) in very widely used herbicide formulations. Accordingly, the toxicity of glyphosate and glyphosate-based formulations (GBF's) has been extensively studied. An earlier extensive review of glyphosate and glyphosate formulation safety and risk assessment included descriptions and analyses of genetic toxicology studies of glyphosate and Roundup™-branded and other glyphosate formulations (Williams et al., 2000). These studies included a wide variety of test systems and endpoints. Subsequent to this review a number of genotoxicity studies of glyphosate and GBF's have been published in the literature. Additionally, there are large numbers of genetic toxicology studies of glyphosate and GBF's sponsored by companies that were not included in the previous review. The number and diversity of these studies warrant careful examination and integration of their findings with the previous results to produce an updated assessment of the overall genotoxicity profile for glyphosate and a genotoxicity profile that is typical of the GBF's.

2. Identification and Analysis of Published Studies

The published studies for review consideration were identified by literature searches for published reports containing references to glyphosate that also contained searchable terms which indicated that genotoxicity studies were performed. Details of search procedures are provided in the online supplementary material. Each identified publication was evaluated to verify that it contained original results of one or more experimental genotoxicity studies on glyphosate or GBF's. Human monitoring studies are not included in this review. Emphasis was

placed on publications in peer-reviewed journals. Abstracts or other sources with incomplete information were not considered. Reviews without original data were not considered for evaluation; however, these reviews were examined to determine if there were any cited publications that had not been detected in the literature searches.

Each relevant publication was examined using several criteria to characterize the scientific quality of the reported genetic toxicology studies. Useful, objective criteria for this purpose were international guidelines for genetic toxicology studies formulated by expert groups. These include principles for conducting studies, reporting results and analyzing and interpreting data. Some of the principles of the guidelines are generally applicable to all studies while others are specific for a particular type of test system and endpoint. Some of the specific types of studies encountered in the review do not yet have international guidelines; however, some of the guideline elements should be generically applicable to these studies. The guidelines for genetic toxicology tests developed for the Organization for Economic Co-operation and Development (OECD) are a pre-eminent source of internationally agreed guidelines. Other international and national guidelines for regulatory genetic toxicology testing are usually concordant with the OECD guidelines. The online supplementary material contains a summary table of some key OECD guideline criteria that were found to be relevant to analysis of the studies considered in this review.

Comparison of the published studies to the criteria in guidelines used for regulatory purposes does not represent an absolute judgment standard but can provide one means for evaluating the quality of the protocols used in the various published studies. Some of the criteria are

rarely met in scientific publications and should be given little or no weight in evaluating the studies. For example, data for individual cultures and individual animals are not commonly included in publications in scientific journals. These data are presumably collected but are usually summarized as group means with a measure of variance for the treatment and control groups. This is not considered to be a significant omission in a scientific publication. However, other guideline features are more essential as scientific quality standards and should be considered as having greater weight in evaluating a study. For example, there are consistent recommendations that assays involving visual scoring (e.g., chromosome aberration, micronucleus and sister chromatid exchange endpoints) should use slides that are independently coded so that scoring is performed without knowledge of the treatment or control group being scored. This guidance is good scientific practice and studies that do not explicitly include a description of coding or "blind" scoring in the methodology would appear to have a deficiency either in the methodology, or perhaps a limitation in the description of the methodology used if coding was actually used and either not indicated or was assumed to be indicated by a reference citation. Other examples of guideline features that have clear experimental scientific value are the use of concurrent negative and positive controls and concurrent measurement and reporting of toxicity endpoints in main experiments, especially in *in vitro* mammalian cell assays.

3. Review and Analysis of Sponsored Regulatory Studies

Reports of genetic toxicology studies sponsored by companies were provided by the companies. The studies were sponsored by companies for regulatory purposes and were conducted at in-house or contract toxicology laboratories. For brevity, the industry sponsored regulatory studies will be subsequently referred to as regulatory studies.

Each study examined was stated to have been conducted in accord with Good Laboratory Practice (GLP) standards with almost all studies citing the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practice (OECD GLP, 1982; OECD GLP, 1997). Reports also cited compliance with various national and regional Good Laboratory Practice Guidelines (e.g., European Commission GLP Directives 87/18/EEC or 88/320/EEC; U.S. Environmental Protection Agency Good Laboratory Practice Standards, 40 CFR Part 160; Japanese Ministry of Agriculture, Forestry, and Fisheries (MAFF) Good Laboratory Practice Standards, 11 Nousan No. 6283). Variations from good laboratory practices were considered not to have significantly impacted the study results.

Almost all of the studies were reported to have been conducted in accord with relevant OECD test guidelines applicable at the time of the study. Study reports were examined to determine that the protocols and experimental methods for the report were consistent with the OECD guidelines and any deviations were noted and considered. Report data were examined to confirm the conclusion of the report regarding whether treatment-related activity had been observed.

4. Glyphosate Structure Activity Analysis

Glyphosate consists of the amino acid glycine joined with a phosphonomethyl group (Figure 1). Glyphosate was evaluated for mutagenic structural alerts using Derek for Windows software (Llhasa Ltd., Leeds, UK, Version 11.0.0, October 24, 2009). No structural alerts were identified for chromosome damage, genotoxicity, mutagenicity or carcinogenicity. The structural components of the glyphosate molecule are not known to be genotoxic; therefore, the lack of structure activity alerts for glyphosate was expected.

5. GBF Compositions

GBF's are herbicide formulations which, by definition, all contain the a.i. glyphosate typically in a salt form (e.g. isopropylamine or potassium glyphosate), but the % glyphosate may be expressed in acid equivalents (a.e.) as percent weight of glyphosate acid without the counter ion. In addition to the a.i., other compounds are included in the formulation to help achieve or improve the herbicidal activity for the desired application. A very common functional component, especially for terrestrial applications, is a compound (or compounds) with surfactant activity that enables better penetration of the a.i. through leaf surfaces. Because formulation compositions are considered proprietary, their specific compositions are not generally indicated in literature reports and are not publicly available for regulatory studies. GBF test materials are usually identified with names or designations and should include either % a.i or a.e. detail.

It should be noted that a common problem encountered in the published literature is the use of the terms "glyphosate", "glyphosate salt" or "Roundup" to indicate any kind of GBF that contains additional components such as surfactants. Published results from studies with different formulations have sometimes been incorrectly or inappropriately attributed to the a.i. The original Roundup™-branded formulation (MON 2139), containing 41% isopropylamine glyphosate salt and 15.4% MON 0818 (a polyethoxylated tallowamine based surfactant blend), is no longer sold in many markets. However, other glyphosate based formulations are sold under the Roundup™ brand name with varying glyphosate forms, concentrations and surfactant systems. Clear identification of the test material is very important in toxicology studies because toxicity of formulations can be dramatically different from the a.i. The fact that test materials identified as Roundup™-branded formulations may actually have different compositions should be considered when comparing results of different studies, as should the possibility that any observed effects may be due to specific GBF components other than the glyphosate active ingredient.

6. Gene Mutation Endpoint

6.1 Bacterial Reversion Assays

6.1.1 Glyphosate and Glyphosate Salts

As reviewed by Williams et al., (2000) six reports of bacterial reversion assays for glyphosate were all negative. No reports of bacterial reversion assays for glyphosate were encountered in the subsequent literature.

A large number of regulatory bacterial reversion assays have been conducted on technical glyphosate and glyphosate salt solutions. These eighteen assays are presented in Table 1. Summary data tables and associated information for the regulatory studies are available in online supplementary material. Methodology and experimental design for these studies was generally in compliance with OECD Guideline 471 (OECD 471, 1997) for studies conducted in or after 1997. The previous guidelines (OECD 471, 1983, for *Salmonella* strains; OECD 472, 1983, for *E. coli* strains) were used for studies conducted before 1997. All of the assays employed a core battery of *S. typhimurium* test strains (TA98, TA100, TA1535 and TA1537 or TA 97a) and most of the assays employed additional *S. typhimurium* TA102 or *E. coli* WP2-derived strains to detect oxidative and cross-linking effects as recommended in OECD 471 (1997). Limitations for some of the studies included three studies using larger than half-log dose level spacing and some studies did not employ a confirmatory assay. One study used positive controls not requiring exogenous metabolic activation for two strains in the presence of S9 (9000xg liver homogenate supernatant). Although this may be considered a deficiency, in that the activity of the S9 was not thoroughly checked, it is only in one of the eighteen studies. The top concentration employed in the assays ranged from 1000 to 5000 µg/plate with most of the studies using the OECD guideline limit dose of 5000 µg/plate. With only a couple of exceptions, the top dose tested produced toxicity as evidenced by thinning of the background lawn, reduction in revertants/plate or both.

None of the studies exhibited revertants/plate exceeding threshold criteria for a positive response: greater than three times the control value for strains with low spontaneous revertants/plate (TA1535 and TA1537) or exceeding two times the control value for the other

strains (Kier et al., 1986). Some studies reported statistical effects. However, none of these cases involved as much as two-fold elevations in revertants per plate and the observations were not consistent with biologically plausible dose responses. In cases with repeated experiments, any increases in revertants/plate were generally not reproducible between experiments~~, the only exception being a small increase in TA100 revertants/plate (less than two-fold over control) reported by Suresh (1993).~~ Therefore, none of the statistically significant effects were judged to indicate mutagenic activity of the test material. Thus, all of the eighteen bacterial reversion studies were concluded to be negative as judged by the absence of significant, reproducible, dose-related increases in revertants/plate. These studies provide abundant weight of evidence that glyphosate and glyphosate salt solutions are negative in bacterial reversion assays under experimental conditions that generally satisfy OECD guidelines

6.1.2 GBF's

As reviewed by Williams et al. (2000) most bacterial reversion studies (Ames/*Salmonella* test strains) for GBF's were negative. Four studies reported negative results for Roundup™-, Rodeo™- and Direct™-branded GBF's. A reported positive Ames/*Salmonella* result for a Roundup™-branded formulation was not replicated in these other studies.

Subsequent to the Williams et al. (2000) review only one published GBF bacterial reversion assay was reviewed (Table 1). This publication reported a negative Ames/*Salmonella* assay result for a GBF of undefined glyphosate composition, Percozyd 10 SL (Chruscielska et al., 2000). Although this result is consistent with the majority of negative Ames/*Salmonella* results for GBF's, the reported study results have significant limitations. One of the recommended test

strains, TA1535, was not used and results were only presented as "-" without presentation of revertants/plate data.

A large number of regulatory bacterial reversion assays have been conducted on GBF's. These are presented in Table 1 with summary data tables in online supplementary material. Methodology and experimental design for these studies was generally in compliance with OECD Guideline 471 (OECD 471, 1997) and with other guidelines. However, two of the studies used some dose level spacings that were larger than the recommended maximum half-log spacing and four studies did not employ a confirmatory assay. All of the assays employed a core battery of *S. typhimurium* test strains (TA98, TA100, TA1535 and TA1537) and employed an additional *S. typhimurium* TA102 or *E. coli* WP2-derived strain to detect oxidative and cross-linking DNA effects as recommended in OECD 471 (1997). The top concentration employed in the assays ranged from 100 to 5000 µg/plate for plate incorporation methodology. With only two exceptions the top dose tested produced toxicity as evidenced by thinning of the background lawn, reduction in revertants/plate or both. For the two exceptions toxicity was noted at higher concentrations per plate in rangefinder assays but toxicity was not noted for the maximum dose selected for the mutagenicity assays.

Only one of the studies exhibited revertants/plate for some strains exceeding up to three-fold of the control value (Mecchi et al., 2003a). However, these increases were not reproducible between experiments and did not exhibit a dose response pattern. These results were therefore judged to be due to low vehicle control revertants/plate and not to indicate treatment-related mutagenic activity. All of the fifteen regulatory bacterial reversion studies

of GBF's were concluded to be negative as judged by the absence of significant, reproducible, dose-related increases in revertants/plate. These studies provide abundant weight of evidence that a variety of GBF's are negative in properly conducted bacterial reversion assays.

6.2 *In Vitro* Mammalian Cell Assays

6.2.1. Glyphosate and Glyphosate Salts

As reviewed by Williams et al. (2000) a CHO/HGPRT *in vitro* mammalian cell gene mutation assay was reported negative for glyphosate when tested up to toxic dose levels of 22.5 mg/mL (≈ 133 mM, millimolar), i.e. well above the current top limit of 10 mM (appropriate for glyphosate and glyphosate salts), in the presence and absence of mammalian metabolic activation.

Two regulatory mouse lymphoma *tk* locus gene mutation studies were reviewed (Table 2 and online supplementary material). One study was conducted according to the 1984 OECD guideline for *in vitro* mammalian gene mutation assays (Jensen, 1991b; OECD 476, 1984). Somewhat fewer cells were exposed (3×10^5 –S9, 1.8×10^5 +S9) than the 10^6 cells recommended in the updated OECD guideline (OECD 476, 1997) but this was not considered a significant deficiency. Cells were exposed at four concentrations up to 4200 µg/mL with S9 (≈24.8 mM) or 5000 µg/mL without S9 (≈ 29.6 mM). Although no toxic effects (reduction in cloning efficiency) were seen on day 0 or day 2, these dose levels exceed the currently recommended upper dose level of 10 mM (1.69 mg/mL for glyphosate) for relatively non-toxic test materials (OECD 476, 1997). It should be noted that most OECD guidelines for *in vitro* mammalian cell genotoxicity assays specify an upper limit dose for soluble, relatively non-toxic

substances of 10 mM or 5 mg/mL, whichever is lower. The lower and appropriate upper limit dose for glyphosate and glyphosate salts is 10 mM. A second study conducted later followed several updated recommendations for *in vitro* mammalian cell gene mutation assays adopted in 1997 (Clay, 1996; OECD 476, 1997). These included the use of at least 10^6 cells in exposed cultures and consideration of test material effects on pH and osmolality. The latter consideration proved to be important because concentrations of 1500 and 2000 µg/mL (≈ 8.8-11.8 mM) produced large (>1 pH unit) decreases in pH and the maximum dose level employed for mutation measurement (1000 µg/mL, ≈ 5.9 mM) was appropriate to avoid excessive effects on pH. This dose level did not produce effects on day 0 cloning efficiency. Although three dose levels were used in the initial experiment, four dose levels (as recommended in OECD 476, 1997) were used in the confirmatory experiment.

Both of the regulatory mouse lymphoma studies were negative for glyphosate when tested up to dose levels that either exceeded the current limit dose or avoided excessive pH effects. These negative results provide important corroboration of a lack of gene mutation activity in the earlier negative CHO/HGPRT study. They also indicate a lack of induction of effects such as large deletions in DNA that may be detected in the autosomal *tk* locus assay (Aaron et al., 1994).

6.2.2 GBF's

No *in vitro* mammalian cell gene mutation assays of GBF's were observed in the published literature or the regulatory study reports.

6.3 Other Non-Mammalian Assays

6.3.1 Glyphosate and Glyphosate Salts

No gene mutation assays on glyphosate other than bacterial reversion or *in vitro* mammalian test systems were reported in Williams et al. (2000) or as regulatory studies. A positive result for glyphosate was reported in the *Drosophila* wing spot assay which can indicate both gene mutation and mitotic recombination endpoints (Kaya et al., 2000). Small increases in small wing spot frequencies were observed in one of four crosses of larvae treated with up to 10 mM (≈1.69 mg/mL) glyphosate. Negative or inconclusive results were observed for the other crosses. The lack of a positive response in the balancer-heterozygous cross offspring, which are insensitive to mitotic recombination events, suggests that there is no evidence for effects on gene mutation endpoint events such as intragenic mutations or deletions in this publication.

6.3.2 GBF's

Williams et al. (2000) described one report of a positive result for a GBF in the *Drosophila* sex-linked recessive lethal assay but this was contradicted by a negative result for the same GBF in this assay reported by another laboratory. Further, the positive study had some features that hampered interpretation, including the lack of concurrent negative controls (Williams et al., 2000). No non-mammalian cell gene mutation assays of GBF's other than bacterial reversion assays were observed in the published literature or the regulatory study reports.

7. Chromosome Effects Endpoints

7.1 *In Vitro* Mammalian Cell Assays

7.1.1 Glyphosate and Glyphosate Salts

Two human and one bovine *in vitro* peripheral lymphocyte chromosome aberration studies of glyphosate were considered in the earlier review (Williams et al., 2000). One human lymphocyte *in vitro* study had negative results for glyphosate tested up to 0.33 mg/mL and 0.56 mg/mL (≈ 2-3 mM) in the absence and presence of an exogenous mammalian activation system, respectively. The other two studies with human and bovine lymphocytes and no metabolic activation system reported positive results at concentrations more than two orders of magnitude lower. The reasons for the conflicting results are unclear, but the Williams et al. (2000) review noted several unusual features about the positive studies including an unusual exposure protocol and discordant positive results for another chemical found negative in other laboratories.

Subsequent to the Williams et al. (2000) review, four publications have reported results for glyphosate salt solutions using cytokinesis block micronucleus (CB MN) or chromosome aberration endpoints with cultured bovine lymphocytes (Table 2). These publications used a test material reported as 62% by weight isopropylamine salt of glyphosate from a Monsanto source. This test material appears to be a manufacturing batch of the isopropylamine salt of glyphosate in water without surfactants, which is not sold as a formulation. In two publications from one laboratory no statistically significant increases in the frequencies of micronucleated binucleate cells were observed following treatment with up to 560 µM (≈94.7 µg/mL acid equivalent, a.e.) for 24 hours in the absence of S9 (Piesova, 2004) or two hours in the absence

and presence of a mammalian metabolic activation system (Piesova, 2005). These two studies both report a statistically significant increase in micronucleus frequency with 48 hours of treatment without S9 in one donor at 280 µM (≈47.3 µg/mL a.e.) but not at 560 µM and in a second donor at 560 µM but not 280 µM. The lack of a consistent response pattern between donors suggests that the results after 48 hours of treatment are questionable. Two other publications found negative results for the chromosome aberration endpoint in cultured bovine lymphocytes with what appears to be the same isopropylamine glyphosate salt solution (Holeckova, 2006; Sivikova and Dianovsky, 2006). Both of these studies used a maximum concentration of 1.12 mM (≈0.189 mg/mL a.e.), which was reported to induce a decrease in mitotic index of >50%, and treatments of 24 hours without S9. These two studies have several limitations including no use of an exogenous mammalian metabolic activation system. In addition, Holeckova (2006) only examined effects detectable by staining of chromosome 1 and apparently did not use a positive control. These four studies consistently indicated lack of chromosome damaging effects in bovine lymphocytes in the absence of metabolic activation following up to 24 hours of exposure to 0.~~51~~256-1.12 mM (≈0.~~087~~094-0.189 mg/mL a.e.) concentrations of glyphosate isopropylamine salt.

Three publications reported testing of technical glyphosate for micronucleus or chromosome aberration endpoints in cultured human lymphocytes (Table 2; Mladinic et al., 2009a; Mladinic et al., 2009b; Manas et al., 2009). The treatment schedule of the Mladinic et al. publications is not clear. Although standard procedures for human lymphocyte assays recommend treatment of exponentially growing cells at 44-48 hours after mitogenic stimulation (OECD 487, 2010), the methodology described in the Mladinic et al. publications suggests that the 4 hour treatment

took place before mitogen stimulation. The cultures were then centrifuged and washed before mitogen was added. Thus, only non-dividing cells would have been exposed and this is clearly not in accord with the OECD guideline. It is also unclear how long the cultures were maintained after treatment. It appears that they may have been cultured for 72 hours after treatment, which suggests the cells would have passed through the required 1.5-2 cell cycles after reaching exponential growth (OECD 487, 2010) even though it appears they were not exposed during exponential growth. Negative or equivocal results for the micronucleus and chromosome aberration endpoints were observed in the absence of exogenous metabolic activation (S9) in all three publications. The maximum exposure concentration in the absence of S9 was in the range of 3-6 mM (≈0.51-1.01 mg/mL) in these studies.

In contrast to the cultured bovine and human lymphocyte results Koller et al. (2012) reported positive results for glyphosate in a CB MN assay using cultured human buccal epithelial cells in the absence of S9. Limitations of this study include no explicit indication of coding of slides or control of pH. However, pH effects would probably not have been observed at the concentrations used. Statistically significant effects were observed at treatment levels of 15-20 mg/L (≈0.09-0.12 mM). Statistically significant effects on nuclear morphology (nuclear buds and nucleoplasmic bridges) were observed at 10-20 mg/L and statistically significant increases in apoptosis and necrosis were observed at 20 mg/L. The concentrations and exposure times reported as producing effects in this study are very substantially lower than the upper dose levels and exposure times used in the previously discussed studies. The results for this discrepancy are not clear, although Koller et al. (2012) suggest that epithelial cells may be more sensitive to the effects of glyphosate than cells of the hematopoietic system such as

lymphocytes. It should be noted that negative genotoxicity results have been observed in a number of regulatory *in vitro* mammalian cell genotoxicity studies using cultured cells other than lymphocytes (mouse lymphoma and CHL cells).

Mladinic et al. (2009a, 2009b) reported increases in micronucleated cells using the cytokinesis-block method in cultured human lymphocytes exposed to glyphosate for 4 hours in the presence of an exogenous human liver metabolic activation system (S9). As discussed above, the methodology used in these studies is unclear, but it appears cells were treated before mitogenic stimulation and cultured for 72 hours. In both publications a statistically significant increase in micronuclei was observed with S9 at the highest dose level of glyphosate tested (580 µg/mL, ≈ 3.4 mM), but how this could be possible when undividing cells were exposed is unclear. Increased proportions of centromere- and DAPI-positive micronuclei were observed for the high dose with S9 suggesting that the induced micronuclei were derived from chromosome loss rather than chromosome fragments. This observation is somewhat unusual, because there do not appear to be any known aneuploidy-inducing agents that require metabolic activation (Kirsch-Volders et al., 2003). Statistically significant increases in the frequency of nuclear abnormalities (buds and bridges) and DNA strand breakage were also observed at the highest dose tested in both publications. In parallel experiments cytotoxic effects such as early apoptosis~~, late apoptosis~~ and necrosis were observed and these effects tended to be enhanced in the presence of S9 (Mladinic et al., 2009a). Also, the negative control levels of such endpoints as necrosis and comet tail moment were significantly increased in the presence of S9 (Mladinic et al., 2009a). It should be noted that glyphosate is mostly excreted unmetabolized *in vivo* in mammals with only very small levels of aminomethylphosphonic acid

(AMPA) or an AMPA-related structure observed (Anadon et al., 2009, Brewster et al., 1991). There is also one report that glyphosate is essentially unmetabolized *in vitro* in the presence of a rat liver S9 homogenate (Gohre et al., 1987). It also does not seem likely that human S9, used by Mladinic et al., would be expected to be more active than much more commonly used induced rat liver S9. These observations suggest that the S9 mediated effects reported by Mladinic et al. are not likely to be due to *in vivo* relevant metabolites. Given the unusual methodology in these studies, the chromosome-damaging effects of glyphosate in the presence of S9 are not convincing, and it is possible that artifacts due to low pH in the presence of S9 (Cifone et al., 1987; Scott et al. 1991; Morita et al. 1992) may be responsible. Such effects would not be relevant to *in vivo* exposures.

Three regulatory *in vitro* mammalian cell chromosome aberration studies were conducted on technical glyphosate (Table 2 and online supplementary material). These studies were conducted in accord with the 1983 OECD Guideline 473 for the *in vitro* mammalian chromosome aberration test (OECD 473, 1983). The study protocols employed exposures in both the presence and absence of an exogenous mammalian metabolic activation system. Treatment and harvest times were appropriate to assess cells exposed in different stages of the cell cycle. Treatment times included a shorter treatment with and without S9 and extended treatments without S9. Appropriate media and culture conditions for these assays were confirmed by experimental results for negative and positive control exposures. ~~One~~ Two of the studies (Wright, 1996; Matsumoto,) used a positive control requiring metabolic activation for the short exposure in the absence of S9. This precluded positive control verification of response for the short exposure in the absence of S9 but was presumably to demonstrate

activity of the metabolic activation system. In these studies slides were coded before analysis, and 200 metaphases per treatment were scored for chromosome aberrations, as recommended in the updated OECD Guideline 473 (OECD 473, 1997). The maximum dose levels used in two of the studies (1250 µg/mL, ≈ 7.4 mM; Wright, 1996; Fox, 1998) were set so as to avoid excessive pH shifts as recommended in the updated OECD Guideline 473. The third study (Matsumoto, 1995) used maximum dose levels (500-1000 µg/mL, ≈ 3-5.9 mM) set by rangefinder results but noted pH-related medium color changes at dose levels of 500 µg/mL and higher.

No induction of chromosome aberrations was observed in these regulatory studies employing cultured Chinese hamster lung cells (two studies) or in two experiments with cultured human lymphocytes from different donors (third study). Taken together these three studies provide clear evidence for lack of *in vitro* mammalian cell clastogenic activity of glyphosate in robust assays for two different mammalian cell types conducted under a variety of exposure conditions in the absence and presence of S9.

The reviewed results for mammalian *in vitro* chromosome aberration and micronucleus assays demonstrate a weight of evidence that technical glyphosate and glyphosate salt concentrates are generally negative for these endpoints in cultured mammalian cells in the absence of an exogenous mammalian metabolic activation system. Three publications from three laboratories and three regulatory studies report negative *in vitro* mammalian cell chromosome aberration or micronucleus results in the absence of exogenous activation. Two publications from one ~~of the laboratories~~laboratory had questionably equivocal results for the micronucleus

endpoint in human lymphocytes in the absence of exogenous activation while two publications from another laboratory reported positive results for bovine lymphocytes with extended treatment but these results did not exhibit a consistent dose response between donors. One publication reported positive results for human ~~tracheal~~ epithelial cells in the absence of S9 with a short exposure time. The negative studies were conducted at upper dose levels and with treatment times that were the same or higher than the studies with positive or equivocal results and include different cell types. These results reinforce the Williams et al. (2000) conclusion that positive chromosome aberration results reported for glyphosate in cultured human lymphocytes in the absence of an exogenous metabolic activation system are not convincing.

Recent reports of positive chromosome aberration and micronucleus results for glyphosate in the presence of an exogenous mammalian activation system in cultured human lymphocytes in one laboratory (Mladinic et al., 2009a, Mladinic et al., 2009b) were not reproduced in three *in vitro* mammalian cell chromosome aberration regulatory studies, including a study that employed cultured human lymphocytes. These positive results are also discordant with one previously reviewed result demonstrating a negative result for glyphosate in cultured human lymphocytes with mammalian metabolic activation using the chromosome aberration endpoint (Williams et al 2000) and a negative result in the presence of S9 for the micronucleus endpoint in bovine lymphocytes (Piesova, 2005). They are also discordant with negative results for three *in vitro* mammalian cell gene mutation studies that included exposure with S9. The unusual methodology used for cultured human lymphocytes in the Mladinic et al. studies further complicates interpretation of the results from these studies. Thus, the weight of

evidence for the *in vitro* chromosome effect assays generally indicates a lack of clastogenic effects in either the presence or absence of S9.

7.1.2 GBF's

No *in vitro* mammalian cell chromosome aberration assays of GBF's are described in Williams et al. (2000).

Only 2 publications with data from *in vitro* mammalian cell chromosome aberration assays of GBF's have been found since the review of Williams et al (2000). Results are in Table 2. Amer et al. (2006) reported positive *in vitro* chromosome aberration effects in mouse spleen cells for a test material described as "herbazed" herbicide, which was reported to contain 84% glyphosate and 16% solvent, an unusually high glyphosate concentration for a formulation. The test material is not further characterized in the publication but is considered a GBF in this review. The glyphosate or GBF concentrations to which the cells in the study were exposed are not entirely clear because the most consistent concentration unit used in the report is M glyphosate/ml which is an unusual concentration unit. Assuming this means moles of glyphosate per mL the maximum exposure would be 5×10^{-5} M glyphosate/mL medium or 50 mM. An upper exposure concentration 50 mM (≈8.45 mg/mL glyphosate) would be well in excess of the limit level of 10 mM or 5 mg/mL currently recommended in OECD guidelines (OECD 473, 1997). In addition to the uncertainty regarding the concentrations used, there are several other limitations to the reported study including no indication that pH of treatment solutions was controlled, no use of a mammalian metabolic activation system and no reported

use of coded slides for scoring. Given these limitations, the uncertainty about the concentrations used and the nature of the test material, these results should not be considered to have significant relevance with respect to typical GBF's.

Another publication reported positive results for Roundup™ Ultra Max GBF for the CB MN assay in cultured human ~~buccal~~ epithelial cells (Koller et al., 2012). Limitations in conduct or reporting of this study included no indication that pH of treatment solutions was controlled and no explicitly reported use of coded slides for scoring. As noted earlier pH effects would not be likely at the low concentrations used. Increased MN frequencies were reported for 20 minute treatments with 10-20 mg/L of glyphosate a.i. (≈ 0.06-0.12 mM glyphosate). Statistically significant effects on nuclear morphology (nuclear buds and nucleoplasmic bridges) were also observed at 10-20 mg/L and increases in apoptosis and necrosis were observed at 20 mg/L but only the necrosis effect was statistically significant.

There were no regulatory studies of GBF's in *in vitro* mammalian cell chromosome aberration or micronucleus assays. Thus, there are only the two studies of different GBF's (discussed above) with uncertainties and limitations in this endpoint category. While the published literature reports suggest the possibility of activity of GBF's in *in vitro* chromosome damage assays, the paucity of studies and their limitations do not permit a generic conclusion regarding this endpoint for *in vitro* mammalian cells for GBF's in general.

7.2. *In Vivo* Mammalian Assays

7.2.1 Rodent Bone Marrow Erythrocyte Micronucleus and Chromosome Aberration

7.2.1.1 Glyphosate and Glyphosate Salts

The Williams et al. (2000) glyphosate toxicity review presented results from *in vivo* mammalian chromosome effect assays. Results from several mouse bone marrow erythrocyte studies of glyphosate were negative for micronucleus induction. These included studies from different laboratories mostly following modern guidelines. The intraperitoneal (i.p.) route was used for most of the negative studies. In addition to i.p. studies, a 13 week mouse feeding study was also negative for the micronucleus endpoint with an estimated maximum daily glyphosate dose of over 11,000 mg/kg body weight/day. There was one published report of a weak positive mouse bone marrow micronucleus response observed for glyphosate. This study, which employed a smaller number of animals per group than other negative studies, clearly conflicted with the numerous other negative studies, not only in terms of increased micronucleus frequencies but also the finding of altered polychromatic erythrocyte to normochromatic erythrocyte (PCE/NCE) ratios. The overall weight of evidence from the earlier reviewed studies was that glyphosate and glyphosate formulations were negative in the mouse bone marrow erythrocyte micronucleus assay. The earlier review also noted a negative mouse dominant lethal result for glyphosate administered by gavage at a maximum dose level of 2000 mg/kg body weight.

As indicated in Table 3, two publications reported results for glyphosate in the mouse bone marrow erythrocyte micronucleus assay. It should be noted that there are some fairly consistent limitations in the reported conduct of these studies compared to OECD guidelines. In these studies concurrent indications of toxicity other than PCE/NCE ratio effects on the bone marrow and mortality are not reported, coding of slides for scoring is not explicitly reported and fewer than the currently recommended number of 2000 PCE's or erythrocytes per animal were scored. As noted earlier, failure to explicitly report coding of slides in the methodology may reflect either failure to code slides or failure to explicitly indicate this in the methodology description in the publication.

Negative results were reported in one study which used a dose of 300 mg/kg body weight of glyphosate administered once i.p. with sacrifices at 24, 48 and 74 hours after dosing (Chruscielska et al., 2000). This study had some limitations including the use of only one dose level (several dose levels should be used except when there is no toxicity up to the limit dose), and no explicit reported coding of slides for scoring and scoring of only 1000 PCE's per animal. A second publication reported positive results for glyphosate administered at 50, 100 and 200 mg/kg body weight via two i.p. injections 24 hours apart, with sacrifice 24 hours after the second dose (Manas et al., 2009). A statistically significant increase in micronucleated erythrocytes was observed in the high dose group in this study. A particular concern with this second publication is that "erythrocytes" rather than polychromatic erythrocytes were indicated as scored for micronuclei. This does not appear to be a case of using "erythrocytes" to mean polychromatic erythrocytes because the term "polychromatic erythrocytes" is used elsewhere in the publication describing measurements of PCE/NCE ratios. Scoring of all

erythrocytes instead of immature polychromatic erythrocytes for micronuclei would be inappropriate in an assay with the stated treatment and harvest times because of the transient nature of micronucleated PCE's in bone marrow (OECD 474, 1997). PCEs containing micronuclei would not have reached maturity in such a short time, so micronuclei in mature erythrocytes could not have been induced by the chemical treatment.

There is no definitive explanation for the discrepancy between the two publications. Although one study used a single dose with multiple harvest times and the second used two doses and a single harvest time, both are acceptable protocols and would not be expected to lead to such discordant results (OECD 474, 1997). The negative result reported for the 13 week feeding study in the earlier review (Williams et al., 2000) confirms that positive results are not simply due to repeat dosing. The reported negative result (Chruscielska et al., 2000) seems to be in accord with a majority of earlier reviewed mouse bone marrow micronucleus studies of glyphosate using similar doses and the i.p. or feeding routes (Williams et al., 2000). Also, the apparent scoring of micronuclei in erythrocytes at such an early time point raises questions regarding the reported positive study.

A large number of regulatory rodent bone marrow assays were conducted on technical glyphosate or glyphosate salt solutions (Table 3 and online supplementary material). Most of these were mouse bone marrow erythrocyte micronucleus studies, but there is also one rat bone marrow erythrocyte micronucleus assay and one mouse bone marrow chromosome aberration study. Most of the rodent bone marrow erythrocyte micronucleus studies were reported to be conducted in accord with OECD Guideline 474 (1983) for studies conducted prior

to 1997 and OECD Guideline 474 (1997) for studies conducted after 1997. The mouse bone marrow chromosome aberration study was reported as conducted according to OECD Guideline 475 (OECD 475, 1984). Protocol features for the micronucleus studies included single dosing with harvest at 24 and 48 hours after treatment (also 72 hours in one study) or two treatments 24 hours apart with a single harvest 24 hours after the last treatment. These treatment and harvest time alternatives are both considered acceptable in the most recent guideline (OECD 474, 1997) for bone marrow erythrocyte studies. For the bone marrow chromosome aberration study, the use of a single 24 hour sampling time after two treatments separated by 24 hours deviates from an earlier recommendation to have 6 hour and 24 hour sampling times with multiple dosing (OECD 475, 1984), but differs only slightly from more recent recommendations to sample approximately 1.5 cell cycles (usually around 12-18 hours) after 2 daily doses (OECD 475, 1997). Some studies used only males when there was no evident difference in toxicity to both sexes, which is acceptable under the most recent guideline (OECD 474, 1997). Three treatment groups were generally used but some studies only used a single high dose group when a limit dose had little or no toxicity as accepted in OECD 474 (1997). In most studies 2000 PCE's per animal were scored as recommended in the most recent guideline (OECD 474, 1997). The earlier guideline had recommended scoring 1000 PCE's per animal (OECD 474, 1983). In the mouse bone marrow chromosome aberration study, 50 metaphases per animal were scored, which is lower than the currently recommended 100 metaphases per animal (OECD 475, 1997).

Eleven mouse and one rat bone marrow erythrocyte micronucleus studies for technical glyphosate or glyphosate salt solutions were conducted. The upper dose levels for orally

administered glyphosate were, with one exception, the earlier suggested limit dose of 5000 mg/kg body weight or the more recently recommended limit dose of 2000 mg/kg body weight. In these studies little or no toxicity was observed at the limit dose. One study (Zoriki Hosomi, 2007) observed considerable toxicity and lethality at an oral dose of 50 mg/kg body weight and employed a lower maximum dose level for the main study (30 mg/kg body weight). The reason for the higher reported toxicity in this study compared to other glyphosate studies is not apparent. Studies of glyphosate employing the intraperitoneal route generally employed lower maximum dose levels (62.5 to 3024 mg/kg body weight) and the maximum dose levels were set by observations of toxicity and lethality in rangefinder studies.

Micronucleated PCE frequency results for the maximum dose levels of the regulatory rodent bone marrow micronucleus studies of glyphosate and glyphosate salts are presented in Table 4. For eight of the twelve regulatory bone marrow erythrocyte micronucleus studies there were no statistically significant increases in micronucleated PCE's observed for any of the glyphosate treated groups. Three studies had small statistically significant increases in micronucleated PCE frequency that were judged not to be treatment related because the frequencies were well within historical control values (Jones, 1999; Durward, 2006; Zoriki-Hosomi).

A statistically significant increase in MN PCE frequency was observed for females treated with 5000 mg/kg in the study of Suresh (1993b). This increase was only about two-fold over the concurrent control and no increase was observed for frequencies of micronucleated normochromatic erythrocytes for this group, although at such an early sampling time this

would not be expected. Historical control data were not presented. Suresh (1993b) employed a high level of glyphosate treatment, 5000 mg/kg body weight, which is well above the currently recommended limit dose of 2000 mg/kg body weight (OECD 474, 1997) as well as an unusual use of groundnut oil as a vehicle for a water soluble test material. The negative control MN PCE frequencies in this study (5.1 and 6.9 MN per 1000 PCE's for females and males, respectively) exceeded control MN PCE frequencies commonly observed in mice (Salamone and Mavournin, 1994). The recommendation by Salamone and Mavournin (1994) is that MN PCE frequencies above 5/1000 MN PCE should be questioned and in most cases confirmed. Two other bone marrow erythrocyte studies which employed 5000 mg/kg body weight treatment did not observe any statistically significant increases in MN PCE frequency (Jensen, 1991; Fox and MacKay, 1996). A mouse bone marrow chromosome aberration study conducted in the same laboratory using the same vehicle and a 5000 mg/kg body weight dose level (Suresh, 1994) was negative. These observations provide a strong weight of evidence that the statistically significant increase observed in Suresh (1993b) is not evidence of a treatment related effect.

The results presented in Table 3 clearly indicate a very strong overall weight of evidence that glyphosate or glyphosate salt solutions do not induce micronucleated PCE's in rodent bone marrow erythrocyte micronucleus assays conducted with maximum dose levels which are appropriate either because of toxic effects or are recommended limit doses for relatively non-toxic compounds. Statistically significant increases in MN PCE frequency in isolated studies were not reproducible in a number of other studies. Furthermore, these studies include several examples of negative results for i.p. administration at maximum doses that exceed those

employed by Manas et al. (2009). It should also be noted that the i.p. route of administration is not relevant to human exposure. In combination with results presented in Williams et al. (2000), there is overall a strong weight of evidence that technical glyphosate and glyphosate salt solutions are not genotoxic in *in vivo* mammalian micronucleus assays at high dose levels.

7.2.1.2 GBF's

The Williams et al. (2000) glyphosate toxicity review presented results from several mouse bone marrow erythrocyte micronucleus studies of GBF's (e.g. Roundup™, Rodeo™ and Direct™-branded formulations) that were mostly negative for micronucleus induction. The intraperitoneal (i.p.) route was used for most of the negative studies and maximum doses for many of the studies were toxic or appropriately close to LD_{50} values. There was one published report of a weak positive mouse bone marrow micronucleus response observed for a Roundup™-branded GBF. This study, which employed a smaller number of animals per group than other negative studies, was clearly aberrant from the numerous other negative studies not only in micronucleated cell frequency finding but also the finding of altered polychromatic erythrocyte to normochromatic erythrocyte (PCE/NCE) ratios. The overall weight of evidence from the earlier reviewed studies was that GBF's were negative in the mouse bone marrow erythrocyte micronucleus assay.

As indicated in Table 3, seven publications reported results for GBF's in *in vivo* mammalian micronucleus or chromosome aberration assays. It should be noted that there are some fairly consistent limitations in the reported conduct of these studies compared to OECD guidelines. In most studies concurrent indications of toxicity other than effects on bone marrow are not reported, coding of slides for scoring is not explicitly indicated and, in many studies, fewer than the currently recommended number of 2000 polychromatic erythrocytes or 100 metaphases per animal were scored.

Three publications report negative results for Roundup™-branded GBF's in mouse chromosome aberration or micronucleus assays. In two of these publications, negative results in mouse bone marrow erythrocyte micronucleus assays were reported for different Roundup™-branded GBF's administered at 200 mg/kg body weight twice 24 hours apart by the i.p. route (Coutinho do Nascimento and Grisolia, 2000; Grisolia, 2002). The third publication reported negative results in mouse bone marrow studies for both the chromosome aberration and erythrocyte micronucleus endpoints using a single oral dose of 1080 mg/kg body weight of a Roundup™-branded GBF (Dimitrov et al., 2006).

In contrast, one publication reported positive results for a Roundup™-branded GBF in mouse bone marrow for the chromosome aberration and erythrocyte micronucleus using a single maximum dose of 50 mg glyphosate/kg body weight i.p. (Prasad et al., 2009). Both the positive results and the magnitude of the increases in the frequencies of chromosome aberrations and micronuclei reported in this study are remarkably discordant with other reported results for Roundup™- branded and other GBF's in mouse bone marrow chromosome aberration and

micronucleus studies in a number of laboratories and publications (Table 3 and Williams et al., 2000). The reasons for this discordance are not clear. One unusual feature of the Prasad et al (2009) study is that the Roundup™-branded GBF was administered in dimethylsulfoxide (DMSO) vehicle. This is an unusual vehicle to use in *in vivo* genotoxicity studies, particularly using the i.p. route and for a test material which is water soluble. A published toxicity study has reported that use of a DMSO/olive oil vehicle by the i.p. route produced dramatically enhanced toxicity of glyphosate formulation or the formulation components without glyphosate compared to saline vehicle (Heydens et al., 2008). The enhanced toxicity observed with this vehicle was not observed when the oral route was used. DMSO has also been shown to enhance the toxicity of other hydrocarbons when administered via the i.p. route (Kocsis et al., 1968). These observations suggest that use of DMSO as a vehicle for administration of chemicals or formulations by the i.p. route might produce unusual toxic effects that are not relevant to normally encountered exposures. Furthermore, the i.p. route is considered by many regulatory agencies to be an unphysiological route and is not recommended for the safety evaluation of chemicals. Regardless of the reasons for the discordant positive results, it is clear that a large preponderance of evidence indicates that Roundup™-branded GBF's are typically negative in mouse bone marrow chromosome aberration and erythrocyte endpoints.

One publication reported positive results for bone marrow chromosome aberration in rabbits administered Roundup™-branded GBF in drinking water at 750 ppm for 60 days (Helal and Moussa, 2005). This study is unique in terms of species and route of administration. The publication does not report water intake in the test and control groups. Given the potential for water palatability issues with a formulated product, this is a significant shortcoming, as any

effects noted might be attributable to dehydration (Saunders, 2005). This study had further limitations including the use of only a single dose level and not explicitly indicating coding of slides for scoring. This study did not include a positive control for chromosome aberration effects. Examination of the chromosome aberration scoring results showed that, for the treated group, large increases were observed for gaps and "centromeric attenuation" which were included in the summation and evaluation of structural chromosome aberration effects. Ordinarily gaps are scored but are not included in the total aberration frequency, and centromeric attenuation is not included in conventional identification of structural aberrations (OECD 475, 1997, Savage, 1976). These unusual scoring and interpretive features raise significant questions about using this study to make conclusions about clastogenicity of the GBF tested.

Two other publications report *in vivo* mammalian chromosome aberration or micronucleus results for non-Roundup™-branded GBF's. In one of these, an uncharacterized GBF, Percozyd 10L, was reported to be negative in a mouse bone marrow erythrocyte micronucleus assay (Chruscielska et al., 2000). The maximum dose level tested, 90 mg/kg i.p., was reported to be 70% of the i.p. LD_{50} as determined experimentally by the authors, and so may have exceeded the maximum tolerated dose. This study had several limitations including use of less than three dose levels and no explicit reported coding of slides for scoring.

In the other study, positive results were reported for another uncharacterized GBF, herbazed, in mouse bone marrow and spermatocyte chromosome aberration studies (Amer et al., 2006) using oral and i.p. routes and treatments from 1 to up to 5 days (i.p.) or 21 days (oral).

Although i.p. exposures of 1, 3 and 5 days produced statistically significant increases in bone marrow abnormal metaphase frequency when gaps were included the increases were not significant excluding gaps and OECD 475 (1997) recommends not including gaps in total aberration frequency. Statistically significant positive results were observed after multiple i.p. exposures (3-5 days bone marrow only including gaps; 5 days for spermatocytes) and after extended oral treatments (14-21 days, bone marrow; 7-21 days spermatocytes) Although not a genotoxic endpoint per se, it should be noted that statistically significant increases in frequency of sperm with abnormal morphology were observed in mice treated with 100 and 200 mg/kg body weight glyphosate p.o. for 5 days. The fact that positive results were not observed in an erythrocyte micronucleus test of mice treated with glyphosate up to 50,000 ppm in feed for 13 weeks (Williams et al., 2000) indicates that, by contrast, extended glyphosate treatment by the oral route does not induce detectable chromosome effects. This treatment was longer and up to much higher glyphosate exposures than those used for the Amer et al. (2006) studies. Thus, it appears likely that these effects were due to some component(s) of the specific herbazed GBF tested rather than glyphosate. It is noteworthy that the Amer et al. (2006) publication is unique in reporting positive responses for such a large number of endpoints for a single test material.

A total of 12 mouse bone marrow erythrocyte micronucleus regulatory studies of GBF's were available (Table 3 and online supplementary material). These studies were designed to be in compliance with OECD 474 (1997) guidance for rodent erythrocyte micronucleus assays. The treatment regimen was either a single oral dose with harvests at 24 and 48 hours after dosing or 2 oral doses 24 hours apart with a single sacrifice at 24 hours after the last dose. Either of these treatment regimens is acceptable under the most recent OECD guideline for this assay

(OECD 474, 1997). Many of the studies used only males but reported no significant differences in gender response in preliminary toxicity studies. All of these studies employed a maximum dose of 2000 mg/kg body weight and most of the studies also used lower doses. This is consistent with a limit dose recommendation of 2000 mg/kg body weight in the OECD guideline. The upper dose level was not reported to induce mortality in any of the studies but in a few studies clinical signs were observed in high dose animals. No toxic effects on bone marrow were generally observed in these studies as judged by PCE/NCE ratios. A decrease in PCE/NCE for 48 hour high dose animals was observed in one study (Xu, 2009a) but this may not have been treatment-related because the control PCE/NCE ratio was unusually high.

Ten of the studies did not exhibit a statistically significant increase in MN PCE for any treatment group. Two studies had a statistically significant increases in MN PCE frequency at the 48 hour time point but the MN PCE frequencies were within historical control levels and judged in each case to be due to a statistical anomaly from a low vehicle control MN PCE frequency and not treatment related (Erexson, 2003a; Xu, 2008a). Thus, none of these twelve studies indicated treatment-related increases in micronucleated polychromatic erythrocyte (MN PCE) frequencies and all studies were considered negative for this endpoint.

In summary, in addition to the *in vivo* rodent bone marrow chromosome effects studies presented in Williams et al. (2000), a majority (three of four) of the rodent bone marrow studies in the subsequent published literature are negative for Roundup™-branded formulations at maximum dose levels that significantly exceed the maximum dose level of the study reporting positive results. One noteworthy feature of the positive study is the use of a

DMSO vehicle which is unusual, if not inappropriate, for a water soluble test material. A rabbit drinking water rabbit study found positive effects for a Roundup™-branded GBF; however, this study had a large number of limitations including not presenting information on palatability and no positive control. Publication reports for other GBF's included a negative study for Perzocyd 10 SL and positive chromosome aberration results for both bone marrow and spermatocytes for a herbazed GBF using extended oral and i.p. treatments. A very large number of well-conducted regulatory mouse bone marrow micronucleus studies indicated that a variety of GBF's are negative in this assay system up to the limit dose of 2000 mg/kg body weight. While the possibility that GBF's with different compositions might have different properties cannot be excluded, the overall data certainly indicate that a typical GBF is negative for induction of chromosome damage in *vivo*.

7.2.2 Rodent Dominant Lethal

The Williams et al. (2000) review notes a negative result in a mouse dominant lethal assay of glyphosate using a maximum treatment level of 2000 mg/kg body weight administered by gavage.

No rodent dominant lethal assays of glyphosate or GBF's were encountered in the subsequent literature.

One regulatory rat dominant lethal study was available (Suresh, 1992; online supplementary material). This study was reported to be conducted in accord with OECD 478 (1984). In this study groups of 30 male Wistar rats were given a single oral administration of glyphosate (suspension in groundnut oil vehicle) at dose levels of 200, 1000 and 5000 mg/kg body weight. Control groups received vehicle only or ethyl methane sulfonate as a positive control. Each week for 10 consecutive weeks males were mated 1:1 to separate groups of untreated virgin females. Each week's paired females were removed after co-housing for six days and were sacrificed on the 16th day after pairing and reproductive parameters were measured (pregnancy status, corpora lutea, early and late resorptions and live implants). One unusual aspect of this study is that mean body weights of all treatment groups were initially statistically higher than the control group mean body weight and this pattern persisted throughout the study. The following effects were observed in the first group of week 1 females mated to high dose males: reductions in pregnancy rate, decreases in live implants and increases in pre and post-implantation loss. There were also increases in embryonic resorptions ("small moles") in week 1 females mated to mid-dose males. These effects were attributed to significant acute toxic effects of glyphosate (not dominant lethal effects) exhibited after treatment in week 1 as evidenced by body weight loss in the mid and high dose males and clinical signs. Although some statistically significant findings in post-implantation loss were sporadically observed in subsequent weeks these were not considered to be treatment related because they were not consistent with a biologically plausible dose response or a biologically plausible time course (see post-implantation loss data table in online supplementary material). This conclusion was also indicated in an EU monograph report (BBA, 1998-2000). This study appears to be in

accord with the study noted in Williams et al. (2000) indicating that glyphosate is not active as a rodent germ cell mutagen.

7.3 Non-Mammalian Assays

7.3.1 Glyphosate and Glyphosate Salts

The Williams et al. (2000) review reported negative results for isopropylamine salt of glyphosate in an onion root tip chromosome aberration assay.

One subsequent published study reported a weak positive result for technical glyphosate in a *Drosophila* wing spot assay (Kaya et al., 2000). Statistically significant positive increases were found only in one of four crosses for small twin spots and not for the two other wing spot categories (large wing spots and twin wing spots). As discussed above, only negative or inconclusive results were observed for crosses that were not subject to mitotic recombination effects. If the result was actually treatment related it only would indicate an increase in recombination events and not in somatic mutations.

7.3.2 GBF's

The Williams et al. (2000) review reported a positive result for a Roundup™-branded GBF for chromosome aberrations in an onion root tip assay and it was noted that this may have been caused by toxic effects of the GBF surfactant.

Negative results were observed in subsequent published *in vitro* assays for the chromosome aberration and micronucleus endpoints in *Crepis capillaris* root meristems exposed to a Roundup™-branded GBF at concentrations up to 0.5% a.i. (Dimitrov et al., 2006).

Subsequent to the earlier review a number of publications have reported discordant results for blood erythrocyte micronucleus assays conducted on GBF's in several non-mammalian fish, reptile and amphibian species (Table 5). One publication reported what might arguably be considered equivocal results for the erythrocyte micronucleus test in *Oreochromis niloticus* (Nile tilapia) administered a test material described as Roundup™ 69 GBF at an upper dose of 170 mg/kg i.p. (Coutinho do Nascimento and Grisolia, 2000). Although there was a statistically significant increase in micronucleated erythrocyte frequency at the mid-dose level, a significant increase was not observed at the high dose level and considerable variability in frequencies in different groups was noted. Negative results were reported in another fish species (*Prochilodus lineatus*) exposed to 10 mg/L Roundup™-branded GBF for 6, 24 and 96 hours (Cavalcante et al., 2008). This concentration was reported to be ~~96~~75% of a 96 hour LC_{50}. Negative results were also reported for the micronucleus endpoint in the fish *Corydoras paleatus* exposed to 6.7 µg/L Roundup™-branded GBF (calculated 3.2 µg/L glyphosate) for 3, 6 and 9 days (de Castilhos Ghisi and Cestari, 2012). Positive results were reported for the erythrocyte micronucleus assay conducted in the fish *Tilapia rendalii* exposed to up to 170 mg/kg body weight i.p. of another Roundup™-branded GBF (Grisolia, 2002). Examination of the micronucleus frequencies in this publication indicated that the negative control micronucleus frequency was considerably lower than the frequencies for all but one of 21 treatment groups for 7 different test materials. This suggests an unusually low control frequency and at least one treatment group had statistically

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significant increases in MN frequencies for each of the 7 test materials. In the absence of historical negative control data and few publications from which to estimate negative control ranges, the possibility that the apparently significant increases were due to a low negative control value should be considered for this publication. Another publication reported positive erythrocyte micronucleus results in goldfish (*Carassius auratus*) exposed to 5 to 15 ppm glyphosate concentration of a Roundup™-branded GBF for 2 to 6 days (Cavas and Konen, 2007).

The reasons for the discordant results are not clear for the fish erythrocyte micronucleus assays of Roundup™-branded GBF's. Although different species and GBF's were used in the different studies there were pairs of studies with positive and negative or equivocal results that used similar treatment conditions (e.g. 170 mg/kg i.p. or 10-15 mg/L in water).

An amphibian erythrocyte micronucleus study reported questionable effects of a Roundup™-branded GBF (Bosch et al., 2011). For one species (*O. cordobae*) toxicity and lethality were observed at exposures to concentrations of 200-800 mg/L a.i. (glyphosate active ingredient) of Roundup™-branded GBF. The surviving 100 mg/L a.i. treatment group had an increase in micronucleated erythrocyte frequency after 5 days but the increase was not statistically significant. A second species (*R. arenarum*) tolerated exposure up to 800 mg/L a.i. Roundup™-branded GBF. No statistically significant differences were found in the ~~treatment~~ experimental groups by analysis of variance. Although a statistically significant correlation between dose and micronucleated erythrocyte frequency was observed at day 2 of treatment this analysis apparently omitted the high dose group which had a mean micronucleus frequency comparable to negative control values. The downturn in dose response and apparent omission of the high

dose from the statistical analysis is peculiar, because significant toxicity was not reported in this species at the 2 day sampling time. The results reported in this publication do not clearly support a conclusion of a micronucleus effect of a GBF in these species.

Results for an unusual test system of exposed caiman eggs are reported in two publications. In one study eggs were topically exposed in a laboratory setting to Roundup™ Full II GBF, and erythrocyte micronucleus formation was measured in hatchlings (Poletta et al., 2009). The GBF tested was reported to contain the potassium salt of glyphosate ~~and alkoxylated alkylamine derivatives as surfactants~~. Statistically significant increases in micronucleated erythrocytes were observed in hatchlings from eggs treated with 500-1750 µg/egg. This system is quite unusual in the species tested and even more so in using an egg application with measurement of effects in hatchlings. Although there is some experience with a hen's egg erythrocyte micronucleus assay using *in ovo* exposure, the erythrocytes were evaluated in embryos only a few days after treatment (Wolf et al., 2008). In the caiman egg assay reported by Poletta et al. (2009) there was presumably a single topical exposure followed by an egg incubation period of about 10 weeks before hatching. It is difficult to envisage that genotoxic events *in ovo* could produce elevated micronucleated erythrocyte frequencies detectable after 10 weeks, given the number of cell divisions occurring in development of a hatchling, and dilution of any micronucleated cells in a larger population as a result of this.

A second publication by Poletta et al. (2011) described two field experiments evaluating caiman hatched from eggs in artificial nests that were sprayed ~~on incubation days 5 and 35. Experiment 1 dosed~~ with ~~two applications of~~ Roundup™ Full II GBF. ~~and~~ A second experiment

~~2, twelve months later, used the same dosing regimen except the second application at incubation day 35~~ included co-treatment with cypermethrin and endosulfan formulations. Increases in micronucleated erythrocyte frequency in hatchlings were reported for both experiments. Additional measurements of growth showed small but statistically significant differences in total length and snout-vent length in 3 month old, but not 12 month old, animals ~~in both experiments~~. Alanine aminotransferase enzyme levels in serum of 3 month old animals ~~in both experiments~~ were significantly elevated (>2-fold control values). Alterations in these parameters suggest that the treated groups had some persistent biological differences or toxic effects either as a result of treatment or some other factor. It is certainly possible that the micronucleus effects in both publications are associated with these persistent biological differences or toxic effects rather than from genotoxic effects induced in the embryos.

There were no regulatory reports of non-mammalian chromosome effect assays.

In summary, the above *in vivo* micronucleus assays in non-mammalian systems have given discordant results for reasons that cannot be precisely defined. Typically these results would be given lower weight than mammalian systems in terms of prediction of mammalian effects, especially since there is very little experience with these systems in comparison with *in vivo* mammalian chromosome effects assays, such as the rat or mouse bone marrow chromosome aberration or erythrocyte micronucleus assays.

8. DNA Damage

8.1 *In Vitro* Mammalian Cell Assays

8.1.1 Glyphosate and Glyphosate Salts

Some positive results for glyphosate for induction of sister-chromatid exchanges (SCE) were reported in cultured human and bovine lymphocytes in the earlier review (Williams et al., 2000). These results tended to be weak, inconsistent and with limited evidence for dose response. A number of limitations were observed for these studies such as the failure to control pH and abnormally low control values. Negative results were reported for technical glyphosate in a *B. subtilis* DNA damage assay and a rat primary hepatocyte unscheduled DNA synthesis assay.

Subsequent to the review there is one publication of a positive *in vitro* SCE result in cultured bovine lymphocytes (Table 6; Sivikova and Dianovsky, 2006). It is noteworthy that negative effects for the chromosome aberration endpoint were reported in this publication.

Positive results for technical glyphosate have been reported for the comet (alkaline single cell gel electrophoresis, alkaline SCGE) endpoint in *in vitro* mammalian cell assays in four publications subsequent to the Williams et al. (2000) review (Table 6). Some general protocol concerns for these studies are failure to explicitly indicate assessment or control of pH or to explicitly indicate coding of slides for scoring. It is possible that these may be deficiencies or limitations in reporting rather than conduct. Positive Comet results were observed for two mammalian cell lines exposed to glyphosate for 4 hours at concentrations of 4.~~5~~0-6.5 mM (≈0.~~76~~68-1.10 mg/mL, GM39 cells) and 4.75-6.5 mM (≈0.80-1.10 mg/mL, HT1080 cells) (Monroy et al., 2005). These concentrations are close to the upper limit dose of 10 mM (appropriate for glyphosate) generally recommended for *in vitro* mammalian cell assays in current OECD

guidelines. Positive Comet results were also reported in Hep-2 cells exposed for 4 hours to 3.~~5~~0-7.5 mM (≈0.~~59~~51-1.27 mg/mL) glyphosate (Manas et al., 2009). This publication reported negative results for the chromosome aberration endpoint in cultured human lymphocytes exposed to up to 6 mM (≈1.01 mg/mL) glyphosate for 48 hours and it should be noted that pH control of the culture medium was reported for the chromosome aberration endpoint. Positive Comet results have also been reported for cultured human lymphocytes exposed to glyphosate at concentrations up to 580 µg/mL (≈ 3.4 mM) for 4 hours (Mladinic et al., 2009a). Effects were observed both in the presence and absence of S9. A modification of the Comet assay employing human 8-hydroxyguanine DNA-glycosylase (hOGG1) to detect oxidative damage only indicated statistically significant effects on comet tail length for 580 µg/mL with S9. ~~Measurements of total antioxidant capacity and thiobarbituric acid reactive substances showed statistically significant increases at 580 µg/mL in the presence or absence of S9.~~ Interpretation of the significance of metabolic activation effects is complicated by the observation that several of the endpoints (e.g., comet tail intensity and nuclear abnormalities) tended to show increases in the presence of S9 in negative controls or at the very lowest concentrations of glyphosate (0.5-3.5 µg/mL, ≈2.9-20.7 µM). ~~A reasonable summation of the results in this publication is that comet effects and other effects such as nuclear abnormalities, early apoptosis, necrosis and oxidative damage were consistently observed at 580 µg/mL.~~ Positive Comet effects were also reported in a human ~~tracheal~~ epithelial cell line at dose levels up to 2000 mg/L (≈ 11.8 mM) (Koller et al., 2012). An unusual feature of these results is that statistically significant increases in comet tail intensity were reported as low at 20 mg/L (0.118 mM) with not much dose response between 40 and 2000 mg/L. These dose levels of glyphosate were observed to

produce little or no effects on a cellular integrity marker but statistically significant effects on necrosis and apoptosis markers were observed at 20 mg/L in parallel experiments.

One regulatory study of technical glyphosate was reported for a primary rat hepatocyte UDS assay (Rossberger, 1994; Table 6 and online supplementary material). In this study cultures of hepatocytes were exposed to glyphosate concentrations of 0.~~07~~2-48.98 mM (≈0.~~012~~34-8.28 mg/mL) and ~~0.13~~1.14-111.69 mM (≈0.~~022~~19-18.88 mg/mL) for 18 hours in two experiments. Radiolabelled and halogen-substituted nucleosides were used to enable replicative and unscheduled DNA synthesis to be identified by density gradient centrifugation and radioactivity counting No effects on unscheduled DNA synthesis were observed in this study in two separate experiments. Measurements of replicative DNA synthesis indicated that cytotoxic concentrations were tested and the maximum concentrations were in any case much higher than recommended for other *in vitro* mammalian cell assays (10 mM for glyphosate). This study is limited by the use of only single cultures per experimental point, although there were two separate experiments. The relatively narrow distribution of repair synthesis values with no dose response in glyphosate-treated cultures, and the clear increases in repair induced by the positive control, suggest that this study provides reasonable evidence for a lack of induced DNA repair following exposure of rat primary hepatocytes to very high concentrations of glyphosate.

Overall there are a number of *in vitro* mammalian cell studies in which glyphosate has been reported to produce positive responses in SCE or Comet assays. Most of these positive responses have occurred at high exposures to glyphosate in the millimolar range. Although lower than the limit dose of 10 mM (appropriate for glyphosate) recommended for several *in*

vitro mammalian cell culture assays (OECD 473, 1997, OECD 476, 1997, OECD 487, 2010), there have been some suggestions that lower dose levels may be more appropriate, particularly because of concerns about relevance of positive *in vitro* findings observed at higher dose levels (ICHS2(R1), 2011, Parry et al., 2010; Morita et al., 2012). In addition, many of the studies have functional limitations such as lack of pH control and no explicit statement regarding coding of slides for visual scoring.

Concerns over the possibility of effects induced by toxicity have led to several suggestions for experimental and interpretive criteria to distinguish between genotoxic DNA-reactive mechanisms for induction of comet effects and cytotoxic or apoptotic mechanisms. One recommendation for the *in vitro* Comet assay is to limit toxicity to no more than a 30% reduction in viability compared to controls (Henderson et al. 1998; Storer et al. 1996; Tice et al. 2000). Importantly, dye exclusion measurements of cell membrane integrity, such as those reported in some of the above publications, may significantly underestimate cytotoxicity that could lead to comet effects (Storer et al., 1996). Other recommendations include conducting neutral diffusion experiments to determine if apoptotic processes might be responsible for comet effects (Tice et al. 2000). ~~Measurement of apoptotic and necrotic incidence performed in one publication (Mladinic et al., 2009a) indicated both apoptotic and necrotic processes generally occurring in parallel with observations of comet effects. Similarly, Koller et al. (2012) reported statistically significant increases in comet effects and apoptosis and necrosis markers at a concentration of 20 µg/L. These observations as well as the reported dose responses, suggest that cytotoxicity frequently accompanies the observations of DNA damage *in vitro* in~~

~~mammalian cells and therefore support the likelihood that the observed effects are secondary to cytotoxicity and are thresholded.~~

In contrast to the SCE and comet endpoints, two independent studies of technical glyphosate in the primary rat hepatocyte UDS assay have both been negative. These results provide evidence that this endpoint is not affected by glyphosate at high concentrations in cell lines with endogenous mammalian metabolic activation capability.

8.1.2 GBF's

Some positive results for glyphosate or GBF's in the sister chromatid exchange (SCE) endpoint were reported in cultured human and bovine lymphocytes in the earlier review (Williams et al., 2000). These results tended to be weak, inconsistent and with limited evidence for dose response. ~~A number of limitations were observed for the studies such as the failure to control pH and abnormally low control values.~~

Subsequent publications of DNA damage assays of GBF's in *in vitro* mammalian cell assays are presented in Table 6. Positive SCE results were observed for the uncharacterized herbazed GBF in mouse spleen cells (Amer et al., 2006). Limitations of this study are in common to those described above (see 6.1) for the chromosome aberration endpoint portion of the study. The magnitudes of the increases in SCE/cell were less than two-fold of the control value which may not be considered biologically significant. Given these limitations, and the fact that the mechanism(s) by which SCE are induced is not understood, these positive findings should be viewed with caution. Koller et al. (2012) reported positive Comet results for human ~~buccal~~ epithelial cells exposed to Roundup™ Ultra Max formulation. Statistically significant effects on

comet tail intensity were observed from exposure to 20-200 mg/L of glyphosate (≈ 0.12-1.18 mM) for 20 minutes.

There were no regulatory DNA damage studies of GBF's in *in vitro* mammalian systems. The Amer et al (2006) report of a positive result for an uncharacterized GBF in the SCE endpoint agrees with other positive findings for this GBF in this publication but because of the discussed limitations does not add significantly to an evaluation of general genotoxic properties for GBF's. Similarly the single observation of comet effects for a different GBF in an *in vitro* cellular assay is of limited value for assessing general GBF properties.

8.2 *In Vivo* Mammalian Assays

8.2.1 Glyphosate and Glyphosate Salts

In the earlier review (Williams et al., 2000), positive results for DNA strand breakage were reported in kidney and liver tissue of mice treated by the i.p. route with glyphosate. The earlier review also noted reports of the absence of DNA adducts in mice treated by the i.p. route with the isopropylamine salt of glyphosate and a possible increase in 8-hydroxydeoxyguanosine in DNA of mice treated with technical glyphosate.

No new *in vivo* mammalian studies of DNA damage or DNA-reactivity of glyphosate were encountered in publications since 2000 and there were no regulatory studies of this category.

8.2.2 GBF's

In the earlier review of Williams et al. (2000) positive results for DNA adducts (^{32}P-postlabelling) and DNA strand breakage were reported for mice treated by the i.p. route with Roundup™ GBF. For a number of reasons these observations were not considered to be clear evidence for DNA reactive genotoxicity of the Roundup™ GBF.

Only one *in vivo* mammalian DNA damage study of a GBF has since been reported. This publication indicated an increase in SCE frequency in bone marrow cells of mice treated with uncharacterized herbazed GBF (Table 6; Amer et al., 2006). Statistically significant positive effects were only observed at the highest dose level tested (200 mg/kg body weight glyphosate administered p.o.) and were less than two-fold of the control value. As noted above, since the mechanism(s) by which SCE's are induced is not understood, this report for one GBF does not add significantly to an evaluation of general genotoxic potential for GBF's.

In a follow-up to ^{32}P-postlabelling, DNA strand breakage and 8-hydroxydeoxyguanosine (8-OHdG) studies cited in Williams et al. (2000) Heydens et al. (2008) reported on studies in mice to further investigate toxic effects and 8-OHdG levels associated with the routes, vehicles and dose levels of the earlier studies. The Heydens et al. (2008) publication reported significant GBF-induced liver and kidney toxicity for high i.p. doses but no liver or kidney toxicity for comparable oral doses. Statistically significant increases in 8-OHdG were not observed in the

latter study under the same conditions as employed by the earlier study. The DMSO/olive oil vehicle dramatically enhanced toxicity of GBF administered by the i.p. route and the toxicity was also observed for formulation components without glyphosate. These results indicated that the effects reported in the earlier studies were associated with high liver and kidney toxicity that was primarily due to the non-glyphosate components of the formulation when administered at very high doses via the i.p. route of exposure. The toxicity enhancement by the unusual DMSO/olive oil dosing vehicle further calls into question whether the ^{32}P-postlabelling finding represented effects associated with unusual toxicity rather than being indicative of adducts formed from glyphosate or glyphosate formulation components.

8.3 Non-Mammalian Assays

8.3.1 Glyphosate and Glyphosate Salts

The Williams et al. (2000) review noted a negative result for glyphosate in the *B. subtilis* H17/M45 rec bacterial differential killing assay.

As presented in Table 7 two subsequent publications reported positive Comet results for glyphosate on Tradescantia flowers and nuclei ([HYPERLINK \l "_ENREF_1" \o "Alvarez-Moya, 2011 #271"]) and negative Comet results for oyster sperm cells exposed to glyphosate (Akcha et al. (2012). The latter study employed a very low maximum exposure of 5 µg/L (≈ 0.03 µM).

There was one regulatory study of technical glyphosate (95.68%) in the *B. subtilis* H17/M45 differential DNA damage (*rec*) assay (Table 7 and online supplementary material; Akanuma, 1995a). This study employed multiple levels of glyphosate on paper disks (up to 240 µg/disk) and measured zones of inhibition. No differential toxicity was observed indicating a lack of genotoxicity in this assay system. This result is in agreement with the earlier reported negative result for this assay in Williams et al. (2000).

8.3.2 GBF's

In the earlier review of Williams et al. (2000), positive results were reported for DNA strand breakage in mouse tissues and for the ~~alkaline SCGE (comet)~~comet endpoint in tadpoles of the frog *Rana catesbiana* exposed to a GBF.

There have been several subsequent publications of results for GBF's in a variety of non-mammalian DNA damage assay systems (Table 7). Two published DNA damage assays *in vitro* reported a positive result for a GBF in the *E. coli* SOS DNA damage test (Raipulis, 2009) and a negative Comet result for oyster sperm cells exposed to a very low (5 µg/L glyphosate, ≈ 0.03 µM glyphosate) concentration of a Roundup™-branded GBF (Akcha et al., 2012).

Several recent publications report Comet results for GBF's in aquatic species (Table 7). Negative Comet results were reported in cells of freshwater mussel larvae exposed to a Roundup™-branded GBF at 5 mg/L (glyphosate a.i.) in water for 24 hours (Conners and Black,

2004). This concentration was reported to be one-half of a no observable effect concentration and the 24-hour LC_{50} for this GBF was reported to be 18.3 mg/L in parallel experiments. Four publications reported positive Comet results in aquatic vertebrates exposed to Roundup™-branded GBF's in water. ~~These publications have a common feature that Comet results were reported as categories of visually damaged cells rather than instrumental measurements of properties such as the DNA tail length or tail intensity~~. In one publication increases in nuclei exhibiting comet visual damage effects were observed in erythrocytes and gill cells of the tropical fish *Prochilodus lineatus* exposed to 10 mg/L of a Roundup™-branded GBF in water (Cavalcante et al., 2008). Measurement of erythrocyte micronucleus frequency and nuclear abnormalities did not show statistically significant increases in these endpoints. A second publication reported positive Comet results in erythrocytes of the goldfish, *Carasseus auratus*, exposed to ~~5, 10 and~~up to 15 ppm glyphosate concentration of a Roundup™-branded GBF for 2, 4 or 6 days (Cavas and Konen, 2007). Similar effects were observed for other endpoints (micronucleus and nuclear abnormalities). Positive comet results were also reported in erythrocytes of the European eel, *Anguilla anguilla*, exposed to 0.058 and 0.116 µg/mL of a Roundup™-branded GBF in water for 1 or 3 days (Guilherme et al., 2010). Increases in nuclear abnormalities were also observed in erythrocytes from animals exposed for 3 days. Positive comet effects were also observed in liver and blood cells isolated from the fish species *Corydoras paleatus* exposed to 0.067 µg/mL Roundup™-branded GBF for 3, 6 or 9 days (de Castilhos Ghisi and Cestari, 2012). No toxicity data other than the absence of mortality were presented but results were negative for the piscine micronucleus endpoint in this study.

8.4 Significance of DNA Damage Endpoint Results

DNA damage endpoints such as SCE or comets are generally regarded as supplementary to the gene mutation and chromosome damage endpoint categories. They are considered indirect measures of genotoxicity. As mentioned above, the precise mechanism(s) behind SCE induction are not understood. DNA damage as measured by Comet assays does not provide information on the consequences of that damage (e.g., repair, mutation or cell death) and such endpoints therefore do not directly measure effects on heritable mutations or events closely associated with chromosome mutations. It is widely recognized that *in vitro* DNA damage endpoints such as the SCE or Comet assay can be induced by cytotoxicity and cell death processes rather than from DNA-reactive mechanisms, as discussed below.

There are numerous examples of SCE positive responses which are unique compared to other genotoxic endpoints, are not concordant with carcinogenicity, or which are induced by oxidant stress (Bradley et al., 1979; Speit, 1986; Benigni, 1989; Zeiger et al., 1990; Eckl et al., 1993; Decuyper-Debergh et al., 1989; Tayama and Nakagawa, 1994; Djelic et al., 2006). These examples indicate that the SCE endpoint, particularly in *in vitro* assays, should not be assumed to indicate DNA reactive genotoxicity or to have the same weight as genotoxicity assays using other endpoints such as gene mutation or chromosome effects.

Similarly, there are abundant data supporting the concept that induction of DNA strand breakage or comet effects can be secondary to necrotic or apoptotic processes that do not involve DNA reactivity (Storer et al., 1996; Henderson et al., 1998; Amin et al., 2000; Hartmann

et al., 2003; Kiffe et al., 2003; Tice et al., 2000; Burlinson et al., 2007). Several clear specific examples exist of *in vitro* induction of comet effects in mammalian cells by conditions which do not appear to be relevant to genotoxic potential at lower doses or which occur by mechanisms that do not involve direct interaction with DNA. These include induction of comet effects by apoptosis inducers which inhibit topoisomerases (Boos and Stopper, 2000; Gieseler et al., 1999); cytokine treatment of cultured cells (Delaney et al., 1997); sodium dodecyl sulfate and potassium cyanide (Henderson et al., 1998); colchicine, dl-menthol and sodium acetate (Kiffe et al., 2003); luteolin (Michels et al., 2005); gossypol (Quintana et al., 2000), carbon tetrachloride (Sasaki et al., 1998) and vitamin C (Anderson et al., 1994). Further examples of induction of comet effects of questionable genotoxic biological significance include dietary flavonoids quercetin, myricetin and silymarin (Duthie et al. 1997); hemoglobin (Glei et al. 2005); olive oil extracts (Nousis et al. 2005) and capsaicin (Richeux et al. 1999).

The observation of effects of sodium dodecyl sulfate is particularly interesting because it suggests responses to surfactants, which are typically components of GBF's. As a more specific example, polyoxyethylenealkalylmine (POEA), a surfactant component of some GBF's, has been shown to elicit cytotoxic effects such as perturbation of the mitochondrial membrane and disruption of mitochondrial membrane potential in cultured mammalian cells (Levine et al., 2007). Surfactant effects provide a very plausible mechanism for observations of GBF's inducing DNA damage responses. Such responses would be expected to be associated with cytotoxic exposures and to exhibit a threshold.

Some data suggest better concordance of the Comet assay with other genotoxic endpoints or carcinogenicity in *in vivo* mammalian studies (Brendler-Schwaab et al., 2005; Hartmann et al., 2004; Kirkland and Speit, 2008). However there are examples of *in vivo* studies of comet effects with questionable significance for genotoxicity because of negative results for other *in vivo* genotoxic endpoints or carcinogenicity assays, or which appear to be due to toxicity. Some examples of non-concordance between comet effects and carcinogenicity include thiabendazole, saccharine, tartrazine and ortho-phenylphenol (Brendler-Schwaab et al., 2005). Discordance between carcinogenicity species specificity and *in vivo* Comet assay results has also been observed (Sekihashi et al., 2002) as well as other positive results for non-carcinogens (Kirkland and Speit, 2008). Another example of questionable *in vivo* genotoxic significance is positive comet effects produced in lymphocytes of exercising humans that were not accompanied by micronucleus induction (Hartmann et al., 1998)

In the context of unique results for DNA damage systems, there are several specific examples of published studies considered in this review containing reported positive results for DNA damage in contrast to negative or equivocal results for chromosome effect endpoints for glyphosate and glyphosate salts in mammalian cells in the absence of S9 (Sivikova and Dianovsky, 2006; Manas et al., 2009; Mladinic et al, 2009a) and GBF's in fish species (Cavalcante et al., 2008; de Castilhos Ghisi and Cestari, 2012).

Concurrent assessment of cytotoxicity is recommended in *in vitro* and particularly *in vivo* studies to assist in interpretation of positive results. The reported "gold standard" for

cytotoxicity in *in vivo* studies is histopathological evaluation of the tissues or cells being evaluated (Burlinson et al., 2007). Other measures for evaluating cytotoxicity include neutral pH SCGE to detect double strand breaks associated with apoptosis or necrosis and measurement of "hedgehogs" which are nuclei in which almost all of the DNA is in the tail (Tice et al., 2000). The latter are thought to represent dead or dying cells severely damaged by cytotoxicity. While "hedgehogs" are usually not included in tabulation of comet effects, they may be used as an additional measure of toxic effects (Smith et al., 2008).

As noted early in Section 8.1, several Comet studies of glyphosate and GBF's did not employ concurrent measures of cytotoxic effects that were optimally suitable for interpretation of a relationship between comet DNA damage and cytotoxicity. Examination of different markers of toxicity in some studies indicated the possibility of association with some markers but not others. The development and routine use of cytotoxicity measurements with maximum relevance to comet effect mechanisms would greatly improve the ability to interpret the significance of this endpoint in both *in vitro* and *in vivo* mammalian systems.

9. Genotoxicity Weight of Evidence Conclusions

The earlier review of Williams et al. (2000) applied a weight of evidence analysis to the available genotoxicity data. Various weighted components included assay system validation, test system species, relevance of the endpoint to heritable mutation, reproducibility and consistency of

effects and dose-response, and relationship of effects to toxicity (Williams et al., 2000). The conclusion of that analysis was that glyphosate and Roundup™-branded GBF's were not mutagenic or genotoxic as a consequence of direct chemical reaction with DNA. This was supported by a strong preponderance of results indicating no effects in *in vivo* mammalian assays for chromosome effects and consistently negative results in gene mutation assays. Although some DNA damage responses were noted, these were judged likely to be secondary to toxicity rather than DNA reactivity.

Since this earlier review, several genotoxicity studies of glyphosate, glyphosate salt solutions and GBF's have been published. Additionally, a large number of unpublished regulatory studies of glyphosate and GBF's were available for this review. A weight of evidence approach was applied to these data that considers the same factors used by Williams et al. (2000) and which are consistent with recommendations for weight of evidence evaluations for genotoxicity data (U.S.EPA, 1986; U.S. FDA, 2006; ICH S2(R1), 2011; EFSA, 2011; UK COM, 2011). Additional considerations include the robustness of the experimental protocols and more recent elaborated considerations relevant to whether genotoxic effects result from direct interaction with DNA or are secondary to other processes such as cytotoxicity (Kirkland et al., 2007; Thybaud et al, 2007).

In terms of composition, the genotoxicity studies of both glyphosate and glyphosate salts can reasonably be considered together to provide an overall evaluation for the glyphosate molecule. This is especially useful when numerous consistent results are observed for a particular endpoint. The fact that glyphosate is present in all GBF's should be considered in

evaluating the genotoxicity of GBF's. It is unlikely that glyphosate or glyphosate salts would contribute novel genotoxic activity (i.e. different from when tested alone) as part of a GBF. Analysis of a weight of evidence of genotoxicity of GBF's should consider the fact that different formulations have different compositions. The weight of evidence, therefore, can allow some conclusions about genotoxicity typical of GBF's but the possibility always exists that individual components could lead to different toxic and genotoxic properties.

Apart from genotoxicity, the data indicate that GBF's are more toxic to the genotoxicity test systems than glyphosate or glyphosate salts, which is consistent with findings in aquatic systems (Folmar et al., 1979; Perkins et al., 2000; Tsui and Chu, 2003). In many cases a reasonable explanation for this difference is that surfactants in the GBF's contribute more to toxicity than glyphosate or glyphosate salts *per se*.

Gene mutation is one of the two primary endpoints with direct relevance to heritable mutation and is considered to be one of the key drivers in the carcinogenic process. A large number of regulatory bacterial reverse gene mutation studies provide a very consistent pattern that glyphosate, glyphosate salts and numerous GBF's are negative in well-conducted GLP regulatory assays.

Additionally, there are two regulatory *in vitro* mammalian cell gene mutation (mouse lymphoma *tk* locus) studies which gave negative results for glyphosate. As noted earlier these mouse lymphoma *tk* locus studies detect large deletions as well as gene mutational events that are also detected in the CHO/HGPRT locus assay. The earlier reported negative CHO/HGRPT

result (Williams et al., 2000) and these negative *tk* mutation results support the conclusion that glyphosate and glyphosate salts do not induce gene mutations in mammalian cells.

The second primary endpoint with direct relevance to heritable mutation and the carcinogenic process is chromosome effects, such as induction of chromosome aberrations or micronuclei in cultured mammalian cells. The earlier review (Williams et al., 2000) noted mixed results for three *in vitro* chromosomal aberration assays for glyphosate, but concluded that the most reliable result was the negative assay. No *in vitro* mammalian cell chromosomal aberration reports were noted for GBF's in the Williams et al. review.

A number of *in vitro* chromosomal aberration and micronucleus assay results for glyphosate or glyphosate salts have been subsequently published using bovine or human lymphocytes. Some technical limitations of these assays were discussed earlier and should be considered in the weight attributed to these studies. Both positive and negative results were reported in these assays. In the absence of exogenous metabolic activation, the majority of studies were negative up to high (mM) dose levels that were toxic or close to toxic levels measured in parallel experiments. Two publications from one laboratory reported an increase in micronucleus frequencies for glyphosate in human lymphocytes in the presence of S9 mix but these studies had several limitations discussed earlier that complicate interpretation of these effects.

A recent publication reported positive CB MN results for glyphosate in cultured human epithelial cells in the absence of metabolic activation at very low dose levels. The dose levels and exposure time reported as producing effects were much lower than dose levels and

exposure times of many published and regulatory *in vitro* mammalian cell genotoxicity studies using different cell types that did not produce either genotoxic or toxic effects. Thus, the results of this study, especially the quantitative aspects, are quite unusual.

Three regulatory chromosomal aberration studies, which used upper dose levels of an estimated 3 mM to around 7 mM, gave negative results in both the presence and absence of S9 ~~mix~~. These results therefore agree with the majority of negative published data in the absence of S9 and support a weight of evidence that glyphosate is not active in *in vitro* mammalian cell gene mutation or chromosome aberration assays in the presence of S9.

Overall, the weight of evidence indicates that glyphosate and glyphosate salts do not typically induce chromosomal aberrations or micronuclei *in vitro* in mammalian cells.

Two publications subsequent to the Williams et al. (2000) review reported positive results for chromosomal aberrations with two different GBF's in two different assay systems. The paucity of studies and study limitations discussed earlier precludes any general conclusion for GBF's for this endpoint. However, as discussed above, the weight of evidence is that glyphosate or glyphosate salts are not clastogenic in mammalian cells, so any positive results with GBF's do not appear to be due to glyphosate.

In vivo mammalian chromosome effect studies are a particularly important class of studies because they are the pre-eminent core assays for *in vivo* mammalian genotoxicity. The Williams et al. (2000) review noted a predominance of negative results for glyphosate in these types of assays with only one study exhibiting a weak positive result.

Two subsequently published studies of glyphosate or glyphosate salt solutions in mouse bone marrow micronucleus assays gave discordant results with one study reporting positive results. However, eight out of twelve regulatory bone marrow micronucleus studies (seven mouse and one rat study) of glyphosate or glyphosate salts did not yield any statistically significant increases in the frequencies of micronucleated PCEs. Three other studies did give statistical increases in MN PCE frequency for high dose levels but these were judged not to be treatment related because they were clearly within the historical negative control range. A fourth study exhibited a statistically significant increase in MN PCE but this result was also judged not to be treatment related by the authors although historical data were not presented. In addition to the micronucleus results, a mouse bone marrow chromosomal aberration study was also negative. There did not appear to be any data to suggest that, in the minority of studies that exhibited some statistical increases in MN PCE frequencies, the effects might be due to factors such as gender, route of exposure or dose level. The clearly negative results from the vast majority of studies, including a large number of robust regulatory studies conducted in accord with good laboratory practices, indicate that, on weight of evidence, glyphosate and glyphosate salts are not genotoxic in rodent bone marrow micronucleus or chromosomal aberration studies.

A preponderance (4/5) of mouse bone marrow micronucleus assays on GBF's were indicated as negative in the earlier Williams et al. (2000) review. Mixed results were observed in subsequent published rodent bone marrow micronucleus or chromosomal aberration studies with a majority (4/6) being negative including 3/4 studies of Roundup™-branded GBF's. One rabbit drinking water study of a Roundup™-branded GBF was positive but there were some

significant limitations of this study, and this is an unusual test model with little or no background data. Another GBF study reported positive results in spermatocytes with extended oral or i.p. treatments. No clear explanation exists for the discordant published mouse bone marrow results such as unique routes or dramatically different maximum dose levels.

The majority of regulatory rodent bone marrow micronucleus studies (11 mouse and one rat study) of various GBF's gave clearly negative results and the two that had statistical increases were also considered negative because the increases were well within historical control values.

The large number of negative regulatory studies, in combination with a majority of negative published studies, indicates that GBF's are generally negative for this important *in vivo* endpoint. The preponderance of negative results for GBF's is also consistent with a weight of evidence that glyphosate or glyphosate salt solutions are negative for chromosomal effects and suggests that formulation surfactant components are also negative for chromosomal effects *in vivo*.

The micronucleus test detects aneugenic as well as clastogenic (chromosome breakage) events. The negative results for the large number of *in vivo* rodent micronucleus studies therefore also support the conclusion that glyphosate, glyphosate salts and GBF's do not induce aneuploidy.

In addition to the rodent bone marrow studies, one regulatory rat dominant lethal study of glyphosate, albeit with some limitations, appears to confirm the earlier negative result for this

type of assay, and reinforces the conclusion that glyphosate is not genotoxic for mammalian germ cells.

Although generally consistent negative results were observed for rodent micronucleus or chromosome aberration assays of GBF's, discordant results were observed in *in vivo* erythrocyte micronucleus studies of fish, amphibians and reptiles. In addition to some technical limitations there is considerably less experience with these assay systems, and consequently these should have less influence in evaluating overall weight of evidence for chromosomal effects.

In general, induction of DNA damage is considered supplementary to induction of gene mutations and chromosomal effects because it does not directly measure heritable events or effects closely associated with heritable events. Regulatory genotoxicity testing focuses on gene mutation and chromosomal effects for initial *in vitro* core testing (Cimino, 2006; Eastmond et al., 2009; ICHS2(R1), 2011; UK COM, 2011; EFSA, 2011).

The Williams et al. (2000) review noted negative DNA damage results for technical glyphosate in the *B. subtilis rec* assay and the primary hepatocyte UDS assay, but noted positive or equivocal results for SCE assays *in vitro* in human or bovine lymphocytes. The negative results for the *B. subtilis rec* and primary hepatocyte UDS assays have been confirmed in subsequent regulatory studies. The UDS result provides information on lack of *in vitro* genotoxic activity when mammalian metabolic activation other than S9 is employed.

Subsequent literature publications indicated several positive responses for *in vitro* mammalian DNA damage endpoint assays of glyphosate or glyphosate salts. These include an SCE response

in bovine lymphocytes and four positive Comet results in cultured mammalian cell lines or human lymphocytes. The positive Comet results were observed in the absence of mammalian metabolic activation and generally at concentrations in the mM range but one publication found positive results at much lower dose levels in human epithelial cells. As noted earlier, observations of differential responses in Comet and chromosomal aberration assays for some of these studies provide some support for the conclusion that the SCE or Comet responses observed may not be predictive of effects on other more relevant endpoints.

The Williams et al. (2000) review noted some equivocal or positive Roundup™-branded GBF results for the SCE endpoint in human lymphocytes and reports of DNA strand breakage in mouse tissues and induction of comets in tadpoles. An observation of mouse liver DNA adducts for a GBF were considered to be of questionable significance. Subsequent literature results for DNA damage in mammalian systems included induction of SCE in cultured mammalian cells and in mouse bone marrow for the uncharacterized herbazed formulation and induction of comets in cultured mammalian cells with a Roundup™ Ultra Max formulation. There were a number of Comet assay reports for GBF's in a variety of aquatic organisms with a preponderance of positive results.

The fact that DNA damage is usually only seen at high, toxic concentrations *in vitro* (e.g., in the 1-10 mM concentration range) or *in vivo* where tissue damage might be induced, suggests that cytotoxic effects rather than DNA interaction may be responsible for the DNA damage reported for glyphosate, glyphosate salts and GBF's. In many Comet assay publications parallel data on toxic effects most directly relevant to comet mechanisms are lacking, and, in addition, many of

the positive DNA damage results have been observed for GBF's in non-standard test systems. It is hoped that clarification of the mechanism and significance of comet effects can be improved by the more routine use of relevant markers such as quantitation of double-strand breaks and hedgehogs and histopathology, as appropriate, for *in vivo* studies. Studies with protocols for specifically identifying surfactant effects would also be useful in clarifying the significance of DNA damage effects of GBF's. However, it seems reasonably clear that GBF's are more toxic than the a.i. and a reasonable conclusion is that consistency of observations of DNA damage, particularly comets, with GBF's might be secondary to toxicity of GBF surfactants.

As discussed extensively in Section 8 there are both general and specific reasons to consider DNA damage assays as subordinate in a weight of evidence for genotoxic risk, especially when they may arise from mechanisms secondary to toxicity. Whatever the precise causes of these DNA damage effects, they do not translate into gene mutations or chromosomal damage as demonstrated by the large preponderance of negative results for glyphosate, glyphosate salts and GBF's in well-conducted bacterial reversion and *in vivo* rodent bone marrow micronucleus assays.

In addition to considering the results relevant to genotoxicity hazard assessment, an important additional perspective on risk can be provided by comparing levels used in experimental studies with expected human levels. For example, estimated margins of exposure between the *in vivo* genotoxicity test systems (e.g., 1000 mg/kg body weight exposure) and calculated systemic doses from an exposure study of farmers (Acquavella et al., 2004; 0.004 mg/kg maximum systemic exposure; 0.0001 mg/kg geometric mean systemic exposure) are in the range of

250,000 for maximum systemic exposure and 10 million for geometric mean systemic exposure. The margins of exposure compared to *in vitro* mammalian cell exposures are also quite large. Assuming uniform distribution, the estimated systemic concentration of glyphosate from the Acquavella et al. (2004) farmer biomonitoring study would be of the order of 24 nM for the maximum and 0.59 nM for the geometric mean exposure. A typical maximum *in vitro* mammalian genotoxicity study exposure of 5 mM represents margins of exposure of 208,000 for the maximum farmer systemic exposure and 8.5 million for the geometric mean farmer systemic exposure~~, respectively~~. Similarly, exposure levels evaluated in several published DNA damage and micronucleus assays in non-mammalian species were conducted at much higher glyphosate concentrations than anticipated under typical environmental conditions. Relevant environmental concentrations representing biologically available glyphosate are not equivalent to application rates. Sorption to soil and sediment occurs following glyphosate applications, significantly diminishing or eliminating glyphosate and POEA surfactant bioavailability to environmental species (Giesy, 2000).

This evaluation of the large volume of genotoxicity data available presents a convincing weight of evidence supporting a lack of genotoxic potential for both glyphosate and typical GBF's in core gene mutation and chromosome effect endpoints. Given this conclusion, and for other reasons discussed, the observation of DNA damage effects seems likely to be secondary to cytotoxic effects. The lack of genotoxic hazard potential evidenced by core gene mutation and chromosome effect studies, coupled with the very low human and environmental species systemic exposure potential discussed above, indicate that glyphosate and typical GBF's present negligible genotoxicity risk.

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Table 1. Bacterial Reversion Assays

Test Material/ Solvent[a]	Strains[b]	S9[c]	Treatment[d]		Com[e]	Results[f]		Ref.
			Method	Maximum		Toxicity	Mutagenicity	
Glyphosate and Glyphosate Salts								
G (98.6%) (W)	0,9,5,7	AR 4% (PI) 6.6% (PR)	PI, PR	2500 µg (-S9) 5000 µg (+S9)	C	T(R)	neg	Jensen (1991a)
G (96.0%) (W)	0,9,5,7,8	AR 10%	PI	1000 µg	>HL, C, P	T(R)	neg[g]	Suresh (1993a)
G (95.68%) (W)	0,9,5,7,PU	PBR 10%	PR	5000 µg	C	T(R)	neg	Akanuma (1995b)
G (95.6%) (D)	0,9,5,7,PK	PNR 10%	PI, PR	5000 µg	C	T(R)	neg[h]	Callander (1996)

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	PUK							
G (95.3%) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T(R)	neg	Thompson (1996)
GK (60%) (W)	0,9,5,7,PK, PUK	PNR 10%	PI, PR	5000 µg	C	T(R)	neg[i]	Callander (1999)
GI (612.7 g/kg) (W)	0,9,5,7a	AR 10%?	PI	5000 µg	>HL, S	T(R)	neg[j]	Ranzani (2000)
G (95.1%) (W)	0,9,5,7,PU	PNR 10%	PI, PR	5000 µg	C	~~PR,~~ T(R)	neg	Sokolowski (2007a)
G (97.7%) (W)	0,9,5,7,PU	PNR 10%	PI, PR	5000 µg	C	~~PI,~~ T(BR) ~~PR, T(R)~~	neg	Sokolowski (2007b)
G (95.0%) (W)	0,9,5,7,PU	PNR 10%	PI, PR	5000 µg	C	~~PI,~~ T(BR) ~~PR, T(R)~~	neg	Sokolowski (2007c)
G (980.1 g/kg) (D)	0,9,-5,7,2	AR ?%	PI	5000 µg	S	N	neg[k]	Ribeiro do Val (2007)

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G (980.5 g/kg) (D)	0,9, 5,7a,2	AR 5%	PI	1000 µg	>HL, S	T (R)	neg[l]	Miyaji (2008)
G (98.8 % w/w) (W)	0,9,-5,7,2	AR 5%	PI, PR	3160 µg	C	T-(BR)	neg[m]	Flugge (2009a)
G (96.66% w/w) (D)	0,9,5,7,PU	PNR 10%	PI, PR	5000 µg	C	T(R)	neg	Sokolowski (2009a)
G (96.3%) (W)	0,9,5.7,PK, PUK	PNR 10%	PI, PR	5000 µg	C	T(R)	neg	Sokolowski (2009b)
G (96.4%) (W)	0,9,-5,-7,2	AR 5%	PI, PR	3160 µg	C	T-(BR)	neg[m]	Flugge (2010b)
G (96.0%) (D)	0,9,5,7, PU	PNR 5%	PI, PR	5000 µg	C	T-(R)	neg	Schreib (2010)
G (982 g/kg) (D)	0,9,-5,-7,2	PNR 5%	PI, PR	5000 µg	C	PI, T(BR) PR, T(R)	neg	Wallner (2010)
GBF's								

Literature Study								
Perzocyd 10 SL (?)[n]	0,9, 7a,2	AR ?%[n]	PI	0.2 µg?[n]	?[n]	?[n]	neg[n]	Chruscielska et al. (2000)
Regulatory Studies								
MON 78239 (33.6%a.e. GK) (W)	0,9,5,7,PU	AR 10%	PI	3330 µg (-S9)[o] 5000 µg (+S9)	C	T (BR)	neg[p]	Mecchi (2003a)
MON 78634 (65.2%a.e. GA) (W)	0,-9,5,7,PU	AR 10%	PI	3330 µg[o]	C	T (BR)	neg	Mecchi (2003b)
FSG 3090-H1 (360 g/l G) (W)	0,9,5,7,2	AR 5%	PI, PR	316 µg (PI, PR+S9) 100 µg (PR – S9)	C	T-(BR)	neg	Uhde (2004)
MON 78910 (30.3%a.e. G) (W)	0,9,5,7,PU	AR 10%	PI	3330 µg (-S9) 5000 µg (+S9)	C	T-(BR)	neg	Xu (2006)

MON 79672 (68.2%a.e. GA) (D)	0,9,5,7,2	AR? 4%	PI	2000 µg	>HL, S	T-(R)	neg	Lope (2008)
MON 79864 (38.7% ~~G~~a.e.) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T-(BR)	neg	Mecchi (2008a)
MON 76313 (30.9% ~~G~~a.e.) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T-(BR)	neg	Mecchi (2008b)
MON 76171 (31.1% ~~G~~a.e.) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T-(BR)	neg	Mecchi (2008c)
Glyphosate liquid formulation (480 g/l GI) (W)	0,9,5,7,2	AR 5%	PI	200 µg	S	N	neg	Camolesi (2009)
MON 76190 (53.2%a.e. GM)	0,9,5,7,2	AR? 4%	PI	2000 µg	>HL, S	T-(R)	neg	Catoyra (2009)

(D)								
MON 79991 (71.6% G) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T (R)	neg	Mecchi (2009a)
MON 76138 (38.5% G) (W)	0,9,5,7,PU	AR 10%	PI	5000 µg	C	T (BR)	neg	Mecchi (2009b)
MON 77280 (495.29 g/L G) (W)	0,9,5,7,2	AR 5%	PI	200 µg	S	N	neg	Camolesi (2010)
TROP M (Glyphosate 480) (484.6 g/l GI) (W)	0,9,5,7,2	AR 5%	PI, PR	1000 µg (PI) 31.6 µg (PR)	C	T (BR)	neg	Flugge (2010a)
Glyphosate granular formulation (76.1% GA) (W)	0,9,5,7,2	AR 5%	PI, PR	100 µg (PI) 10 µg (PR)	C	T (BR)	neg	Flugge (2010d)

[a] Test material and solvent used: G, glyphosate technical (acid); GK potassium salt of glyphosate; GI, isopropylamine salt of glyphosate; GA, monoammonium salt of glyphosate. First entry in () for glyphosate or glyphosate salts indicated purity or concentration. First entry in () for GBF's indicates active ingredient, if indicated, and ingredient concentration. a.e. after % indicates concentration is in acid equivalents. Second entry in () indicates test material solvent: (W), water; (D), dimethyl sulfoxide.

[b] Test strains used: 0, TA100; 9, TA98; 5, TA1535; 7, TA1537;7a, TA97a; 2, TA102; 8, TA1538; PU, E. coli WP2 (uvrA); PUK, E. coli WP2 [pKM101]; PK, E. coli WP2 [pKM101]

[c] S9 metabolic activation system: AR, Aroclor-induced rat liver; PNR, phenobarbital and napthoflavone induced rat liver; PBR, phenobarbital and benzoflavone induced rat liver; percentage number indicates percentage of S9 in S9 Mix.

[d] Treatment Conditions: Method--treatment methodology: PI, plate incorporation; PR, preincubation. Maximum--maximum amount per plate tested. In some cases differences between treatment conditions were used as indicated.

[e] Comments on assay:. >HL, more than half-log (√10) for one or more dose intervals; C, confirmatory experiment reported; S, single experiment reported; P, positive controls that didn't require S9 metabolic activation ~~for two strains (TA1535 and TA1537)~~ were used for two strains (TA1535 and TA1537) with S9 ~~that didn't require S9 metabolic activation~~.

[f] Results reported for:

Toxicity: T, toxic effects at maximum concentration or lower for one or more strains; (R), reduced revertants/plate; (B), reduced background lawn; (BR), reduced revertants/plate and background lawn; N, no toxic effects.

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Mutagenicity: overall judgment of assay result for test material: neg, negative; individual study increases in revertants/plate or statistical findings are indicated as individual footnotes.

[g] Statistically significant increase for TA100 (+S9) reported in text but not indicated in data tables. Increases were less than 2-fold over control and judged not to indicate a treatment related effect

[h] Statistically significant increases in revertants/plate ~~in one experiment~~ for TA100 +S9, WP2 [pKM101] +S9, TA98 –S9; WP2 (pKM101) –S9. Increases were less than two fold, not reproducible in separate experiments and not consistent with a dose response (e.g. occurring at mid dose levels). Increases were less than 2-fold over control and judged not to indicate a treatment related effect

[i] Statistically significant increases in revertants/plate ~~in one experiment~~ for TA100 –S9, TA1535 –S9, TA98 +S9, TA100 +S9, TA1535 +S9, WP2 [pKM101] +S9~~,~~ WP2[pKM101] –S9 and WP2*uvrA*[pKM101] –S9. Increases were judged not significant because they were all less than two-fold over control values, not reproducible and not consistent with a dose response.

[j] Statistically significant increases in revertants/plate for TA98 +S9 and TA100 +S9 ~~and statistically significant dose responses for TA97a –S9, TA98 +S9 and TA 1535 +S9~~. Increases ~~and dose responses were~~ judged not to indicate treatment-related effects because they were all less than two-fold and not consistent with a dose response~~-~~.

[k] Statistically significant ANOVA with increases for lowest dose levels for TA1537 +S9. Increases judged not to indicate a treatment related effects because they were less and two-fold and not consistent with a dose response.

[l] Statistically significant increases for TA98 +S9 (low to mid-doses) and for TA100 +S9 at one dose. Increases were judged not to indicate a response because they were less and two-fold and not consistent with a dose response

[m] Statistical analysis suggested in text but not clearly evident in data tables.

[n] Not clearly indicated in publication. Numerical data for revertants/plate not presented but summarized as "-" for lack of mutagenic activity.

[o] 5000 µg/plate maximum dose level for WP2*uvrA* –S9 and in one experiment for TA98 and TA1535 –S9 (Mecchi, 2003a).

[p] Several dose levels exceeded control revertants/plate by more than three fold in one experiment for TA98 -S9 and TA1535 -S9. There was no dose response and the result was not observed in a second experiment. The result was considered due to a low control values rather than a treatment-related response.

Table 2. ***In Vitro*** **Mammalian Cell Assays of Glyphosate, Glyphosate Salt Solutions and GBF's**

Test Material[a]	Endpt[b]	Cell type[c]	Treatment[d]							Results		Ref.
			S9[e]	Time[f]		Dose levels/ Replicates./ Ind. expts.[g]	Maximum Dose[h]	pH[i]	Score[j]	Tox[k]	Mut[l]	
				-S9	+S9							
Gene Mutation												
Glyphosate and Glyphosate Salts												
Regulatory Studies												
G (98.6%) (M)	TK	ML		4 (48)		4/2/C	5000 µg/ml (≈29.6 mM)	NI	NA	CE-	neg	Jensen (1991b)
			AR 30%		3 (48)	4/2/C	4200 µg/ml (≈24.8 mM)	NI	NA	CE-	neg	
G (95.6% w/w) (D)	TK	ML	PNR 5%	4 (48)	4 (48)	3 and 4/2/C	1000 µg/ml (≈5.9 mM)	pH	NA	CE-	neg	Clay (1996)

Chromosome Aberration or Micronucleus												
Glyphosate and Glyphosate Salts												
Literature Studies												
GI (62%) (W)	CB MN	BL	none	24		5/1?/C	.56 mM	NI	1000BN (NC)	CBPI-	neg	Piesova (2004)
	CB MN	BL	none	48		5/1?/C	.56 mM	NI	1000BN (NC)	CBPI-	equiv[m]	
GI (62%) (W)	CB MN	BL	10% AR	2 (48)	2 (48)	5/1?/C	.56 mM	NI	1000BN (NC)	CPBI-	neg	Piesova (2005)
	CB MN	BL	none	48		5/1?/C	.56 mM	NI	1000BN (NC)	CPBI-	equiv[m]	
GI (62%) (W)	CA (1)	BL	none	24		6/1?/C	1.12 mM	NI	350-900M (NC)	NI	neg[n]	Holeckova (2006)
GI (62%) (W)	CA	BL	none	24		6/1?/C	1.12 mM	NI	100M (NC)	MI+	neg	Sivikova and Dianovsky (2006)
G (96%) (M)	CA	HL	none	48		3 (>HL)/1?/C	6 mM	pHa	100M	MI-	neg	Manas et al.

												(2009)
G (98%) (P)	CB MN	HL		4 (72?)[n]		5 (>HL)/2/C	580 µg/ml (≈3.43 mM)	pHa	1000BN (NC)	EA+ NE+ NB+ CPBI-	equiv[o,p]	Mladinic et al. (2009a)
	CB MN	HL	H 10%		4 (72?)[n]	5 (>HL)/2/C	580 µg/ml (≈3.43 mM)	pHa	1000BN (NC)	EA+ NE+ NB+ CPBI-	pos[o]	
G (98%) (P)	CB MN	HL		4 (72?)[n]		5 (>HL)/2/C	580 µg/ml (≈3.43 mM)	pHa	2000BN (NC)	CPBI- NB+	equiv[o,q]	Mladinic et al. (2009b)
			H 10%		4 (72?)[n]	5 (>HL)/2/C	580 µg/ml (3.43 mM)	pHa	2000BN (NC)	CPBI- NB+	pos[o]	
G (95%)	CB MN	TR146	none	20 min. (48)		3/3/S	20 mg/L (≈0.12 mM)	NI	>3000B N (NC)	AP+ NE+ NB+	pos	Koller et al. (2012)
Regulatory Studies												

G (95.3% w/w) (M)	CA	CHL	AR 5%	6 (24)[r], 24, 48	6 (24)	3/2/S	1250 µg/ml (≈7.39 mM)	pH	200M	RG-	neg	Wright (1996)
G (95.6%) (M)	CA	HL	PNR 25%	20	3 (20)	3 (>HL)/2/C	1250 µg/ml (≈7.39 mM)	pH	200M	MI+ (-S9)	neg	Fox (1998)
				44	3 (44)	1/2/S	1250 µg/ml (≈7.39 mM)	pH	200M	MI-	neg	
G (95.68%) (H,M)	CA	CHL	PBR 10%	6 (24)[t]	6 (24)	3/2/S	1000 µg/ml (≈5.92 mM)	pHn	200M	MI-	neg	Matsumoto (1995)
				24		3/2/S	500 µg/ml (≈2.96 mM)	pHn	200M	MI-	neg	
				48		3/2/S	500 µg/ml (≈2.96 mM)	pHn	200M	MI-	neg	
GBF's												
Literature Studies												
herbazed	CA	MS	none	24		3 (>HL)/5/S	50 mM[s]	NI	100M	VC	pos	Amer et al. (2006)

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(84% G) (M)												
Roundup™ Ultra Max (450 g/l G) (M)	CB MN	TR146	none	20 min (48)		3/3/S	20 mg/L glyphosate (≈0.12 mM)	NI	>3000B (NC)	AP+ NE+ NB+	pos	Koller et al. (2012)

[a] Test material and solvent used : G, glyphosate technical (acid); GK potassium salt of glyphosate; GI, isopropylamine salt of glyphosate; GA, monoammonium salt of glyphosate. First entry in () for glyphosate indicates percent purity or concentration. First entry in () for GBF's indicates active ingredient and ingredient concentration. Second () entry indicates test material solvent: (W), water; (D), dimethyl sulfoxide, (M), culture medium (H), Hanks balanced salt solution.

[b] Assay endpoint: TK, gene mutation at the TK locus; CA, chromosome aberration; CA (1), chromosome aberration (FISH analysis of chromosome 1 for acentric fragments); CB MN, cytokinesis block micronucleus

[c] ML, L5178Y mouse lymphoma; CHL, Chinese hamster lung; HL, human peripheral blood lymphocytes; BL, bovine peripheral blood lymphocytes; TR146, human buccal epithelial cell line; MS, mouse spleen cells

[d] In cases where treatments differ in the presence and absence of exogenous metabolic activation treatment parameters are presented on separate lines.

[e] Type of S9 used with %S9 homogenate in S9 Mix indicated in (): AR, Aroclor-induced rat liver; PNR, phenobarbital/naphthoflavone-induced rat liver; PBR, phenobarbital/5,6-benzoflavone-induced rat liver; H, human liver; ?, S9 not clearly indicated; none, no experiments conducted with exogenous mammalian metabolic activation.

[f] Duration of treatment in hours with total time or times to harvest in hours from treatment in () if treatment was not continuous.

[g] First number: number of analyzable treatment dose levels with (>HL) indicating spacing between one or more treatment levels greater than half-log; second number: number of replicates cultures for each treatment; third character: C, confirmatory experiments reported for cell lines or multiple donors for lymphocytes; S, no confirmatory experiment reported.

[h] Maximum dose level tested and scored with calculated mM in () for glyphosate.

[i] Assessment or consideration of pH effects of test material: NI, no measurement or control of pH reported; pH, large pH effects noted at higher concentrations and maximum set to minimize pH effects; pHn, effects on pH noted but not used to set maximum treatment concentration; pHa, pH adjusted.

[j] Number of cells or metaphases scored per treatment level/time point for chromosome aberration and micronucleus assays. M, metaphases; BN, binucleated cells. (NC) indicates that coding of slides for scoring was not explicitly indicated. In some cases coding was not explicitly indicated but may have been implied by a reference citation. NA, not applicable.

[k] Measurement of cytotoxicity with + indicating effects on endpoint at one or more treatment levels and -indicating no effects on endpoint up to maximum treatment level: CE, cloning efficiency; RG, relative growth; MI, mitotic index; CPBI, cytokinesis block proliferation index; EA, early apoptosis; NE, necrosis ; AP, apoptosis; NB, nuclear buds; LDH, LDH release (cell integrity); NR, neutral red (vital stain); VC, viable cell staining; NI, no concurrent cytotoxicity measurement reported.

[l] Evaluation of mutagenicity or chromosome effects: neg, negative; pos, positive; equiv, equivocal. Evaluation different from publication or report indicated with individual footnote.

[m] Statistically significant increases observed at a single different dose for each of two donors. Publications indicate dose responses were not observed and effects were weak or minimal with 48 hours treatment.

[n] No positive control reported.

[o] Lymphocytes apparently treated before exposure to mitogenic stimulus.

[p] Small increases in MN frequency in binucleate cells observed for a wide range of dose levels (3.5-580 µg/mL) but no statistically significant increases were observed and no dose response statistic was reported.

[q] No statistically significant increases in MN frequency for any dose level. Statistically significant correlation observed betweeen dose and MN frequency but approximately the same very small increase was observed over a very wide range of doses (3.5-580 ug/mL) and this is considered to be questionable as a biologically plausible dose response.

[r] Positive control requiring metabolic activation ~~(cyclophospharmide)~~ used for 6 hour exposure without S9.

[s] Calculated from the stated concentration of 5×10^{-5} M glyphosate/mL.

Table 3. *In Vivo* Mammalian Chromosome Effect Studies

Test Material[a]	Endpt[b]	Strain/ Species	Treatment[c]						Scoring[d]	Results[e]		Ref.
			Veh	Rte	No/Sex	Grps	Schedule	Maximum Dose		Tox	Mut	
Glyphosate and Glyphosate Salts												
Literature MN Studies												
G	BM MN	C3H mice	W	i.p.	6M	1	S (24, 48C, 72)	300	1000P (NC)	M-, R-	neg	Chruscielska et al. (2000)
G (96.0%)	BM MN	Balbc mice	S?	i.p.	5M 5F	3	T (24)	200	1000E(NC)	M-, C-, R-	pos[f]	Manas et al. (2009)
Regulatory MN Studies												
G (98.6%)	BM MN	NMRI SPF mice	0.5% CMC	p.o.	5M 5F	1	S (24, 48, 72)	5000	2000P *N	M-, R-	neg	Jensen (1991c)

G (96.8%)	BM MN	Swiss mice	PO	p.o.	5M 5F	3 (>HL)	T (24)	5000	≈2000E ≈1000P (NC)	M-,C-, R-	neg[g]	Suresh (1993b)
G (95.6% w/w)	BM MN	CD-1 mice	PS	p.o.	5M 5F	1	S (24, 48)	5000	2000P	M-, C-, R-	neg	Fox & Mackay (1996)
GK (59.3%)	BM MN	CD-1 mice	W	p.o.	5M	1	S (24, 48)	2000	2000P	M-, C-, R-	neg[h]	Jones (1999)
G (954.9 g/kg)	BM MN	Swiss albino mice	W	i.p.	5M 5F	3	T (24)	562.5	1000P 1000N	M-, R-	neg	Marques (1999)
GI (612.7 g/kg)	BM MN	Swiss albino mice	W	i.p.	5M[i] 5F[i]	3	T (24)	3024	1000P *N	M+, R-	neg	Gava (2000)
G (97.73%)	BM MN	NMRI mice	PEG 400	p.o.	5M 5F	3	S (24, 48 H)	2000	2000P	M-, C-, R-	neg	Honarvar (2005)
G (95.7%	BM MN	Crl:CD-	PBS	i.p.	7M	3	S (24, 48 CH)	600	2000P	M-, C+,	neg[j]	Durward (2006)

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w/w)		1®(ICR) BR mice								R+		
G (980.1 g/kg)	BM MN	Swiss mice	W	p.o.	6M	3	T (24)	30	3000P	M-, R-	neg[k]	Zoriki Hosomi (2007)
G (99.1% w/w)	BM MN	NMRI mice	0.5% CMC	p.o.	5M	3 (24 h) 1 (48 h)	S (24, 48 CH)	2000	2000P	M-, C-, R-	neg	Honarvar (2008)
G (980.0 g/kg)	BM MN	Swiss albino mice	CO	i.p.	5M 5F	3	T (24)	62.5	2000P *N	M-, R-	neg	Costa (2008)
G (98.8% w/w)	BM MN	Crl(CD)(SD) rats	0.8% HPMC	p.o.	5M 5F	3	S (24, 48 CH)	2000	2000P	M-, C-, R-	neg	Flugge (2009b)
Regulatory CA Study												
G (96.8%)	BM CA	Swiss albino	PO	p.o.	5M 5F	1	T (24)	5000	50M	M-, C+,	neg	Suresh (1994)

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		mice								MI-		
GBF's												
Published Studies												
Perzocyd 10 SL	BM MN	C3H mice	W	i.p.	6M	1	S (24, 48C, 72)	90	1000P NC	M-, R-	neg	Chruscielska et al. (2000)
Roundup 69	BM MN	mice	NI	i.p.	6M	3	T (25)	200	1000P 1000N NC	M-, R-	neg	Coutinho do Nascimento and Grisolia (2000)
Roundup (480 g/l GI)	BM MN	Swiss mice	W?	i.p.	8M 8F	3	T (24)	200	2000E(P) NC	M-, R-	neg	Grisolia (2002)
Roundup (480 g/l GI)	BM CA	New Zealand white rabbits	W	d.w.	5M	2[l]	60 days	750 ppm	50M NC	M-	pos	Helal and Moussa (2005)
Herbazed (84% G)	BM CA	Swiss mice	NI	i.p.	5M	1	1, 3, 5d (24h)	50 gly	100M NC	M-	equ[m]	Amer et al. (2006)
Herbazed	SC CA	Swiss mice	NI	i.p.	5M	1	1, 3, 5d (24h)	50 gly	100M	M-	pos	

(84% G)									NC			
Herbazed (84% G)	BM CA	Swiss mice	NI	p.o.	5M	2	1, 7, 14, 21d (24)	100 gly	100M NC	M-	pos	
Herbazed (84% G)	SC CA	Swiss mice	NI	p.o.	5M	2	1, 7, 14, 21d (24)	100 gly	100M NC	M-	pos	
Roundup	BM CA	C57BL mice	W	p.o.	8M	1	S (6, 24, 48, 72, 96, 120)	1080	50M	M-	neg	Dimitrov et al.(2006)
	BM MN	C57BL mice	W	p.o.	8M	1	S (6, 24, 48, 72, 96, 120)	1080	500P	M-, R-	neg	
Roundup (41% GI)	BM CA	Swiss mice	DMSO	i.p.	5M	2	S (24, 48, 72)	50 gly	75M NC	M-, MI+	pos	Prasad et al. (2009)
	BM MN	Swiss mice	DMSO	i.p.	5M	2	S (24, 48, 72)	50 gly	2000P NC	M- MI+	pos	
Regulatory Studies												
MON 78239 (36.6%a.e. GK)	BM MN	Crl:CD-1®(ICR) BR mice	W	p.o.	5M	3	S (24, 48 CH)	2000	2000P	M-, C-, R-	neg[n]	Erexson (2003a)

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MON 78634 (65.2% G)	BM MN	Crl:CD-1®(ICR) BR mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Erexson (2003b)
MON 78910 (30.3% G)	BM MN	CD-1I®(ICR)BR mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Erexson (2006)
MON 79864 (38.7% G)	BM MN	Hsd:ICR(CD-1 mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C+, P-	neg[n]	Xu (2008a)
MON 76171 (31.1% G)	BM MN	CD-1®(ICR)BR mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Xu (2008b)
MON 79991 (71.6% G)	BM MN	CD-1®(ICR)BR mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R+?	neg	Xu (2009a)
MON 76138 (38.5% G)	BM MN	CD-1®(ICR)BR mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Xu (2009b)
MON 76313 (30.9% G)	BM MN	Hsd:ICR(CD-1) mice	W	p.o.	5M	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Xu (2009c)

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A17035A (280.7 g/L G)	BM MN	Swiss mice	W	p.o.	6M	1	T (24)	2000	3000P	M-, C-, R-	neg	Negro Silva (2009)
TROP M (483.6 g/l GI)	BM MN	NMRI mice	.8% CMC	p.o.	5M 5F	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Flugge (2010c)
Glyphosate 757 g/kg formulation (69.1%a.e. GX)	BM MN	Crl(CD)(SD) rat	0.8% HPMC	p.o.	5M 5F	3	S (24, 48CH)	2000	2000P	M-, C-, R-	neg	Flugge (2010e)
Glyphosate SL (499.35 g/L G)	BM MN	Swiss mice	W	p.o.	6M	1	T (24)	2000	3000P	M-, C-, R-	neg	Negro Silva (2011)

[a] G, glyphosate technical acid; GK, potassium glyphosate salt; GI, isopropylamine glyphosate salt; GX, unspecified salt; () indicates purity or concentration for glyphosate or glyphosate salts or active ingredient content for GBF's.

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[b] Endpoint: BM MN , bone marrow erythrocyte micronucleus; BM CA, bone marrow chromosome aberration; SC CA, spermatocyte chromosome aberration.

[c] Treatment

Veh--Vehicle used: W, water; S, saline; PO, peanut oil; PS, physiological saline; PEG 400; PBS, phospate buffered saline; CO, corn oil; HMC, DMSO, dimethylsulfoxide; CMC, carboxymethylcellulose; HPMC, hydroxypropylmethylcellulose; NI, not indicated.

Rte--Route of administration: p.o. oral (gavage); i.p., intraperitoneal injection; d.w., drinking water.

No/Sex-- Number of males (M) and females (F) scored for each glyphosate or GBF treatment group.

Grps--Number of glyphosate or GBF dose level treatments scored for micronuclei or chromosome aberrations. >HL indicates spacing between one or more treatment groups greater than half-v10.

Schedule--Treatment schedule for glyphosate treatments: S, single treatment; T, two treatments 24 hours apart; d, consecutive days of treatment with a separate group for each number of days. Numbers in parentheses are harvest times in hours after treatment or last treatment with a separate group for each harvest time. Treatment or harvest conditions used specifically for other groups are indicated as C, vehicle control, H, high dose.

Maximum dose --Maximum glyphosate or GBF treatment dose level in mg/kg body weight except for ppm which indicates amount in drinking water. gly for GBF's indicates that dose units were reported as mg/kg body weight of glyphosate.

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[d] Number indicates cells or metaphases scored per animal for P (PCE's), N (NCE's), E (erythrocytes), M (metaphases). *N, variable NCEs scored for micronuclei while scoring the indicated number of PCEs. E(P) indicates number of erythrocytes scored with results for PCE's reported separately. NC, coding of slides for scoring not explicitly indicated in report or publication. In some cases coding was not explicitly indicated but may have been implied by a reference citation.

[e] Results

Tox--Measures of toxicity reported—M, mortality; C, clinical signs; R, PCE/NCE ratio; MI, mitotic index . A "+" after the measure indicates treatment-related effects. A "-" after the measure indicates no treatment-related effects; +? Indicates a decrease in (R) but control (R) value for the corresponding time point was unusually high. No mortality (MI-) was assumed unless mortality was indicated.

Mut--Overall evaluation of study results as negative (neg), positive (pos) or equivocal (equ) for treatment-related effects. Individual footnotes are used to indicate statistically significant effects or difference with conclusion of publication or report authors.

[f] Statistically significant increase reported for micronucleated erythrocytes. Results not reported for micronucleated PCE's.

[g] Statistically significant increase in MN erythrocytes for high dose females. Not judged to be treatment related.

[h] Statistically significant increase in MN PCE frequency at 24 h only, within historical control, not judged to be treatment-related.

[i] Only 4 males and 4 females scored for high dose group .

[PAGE * MERGEFORMAT]

[j] Statistically significant increase in MN PCE frequency only for 24 h high dose, within historical control , not judged to be treatment-related.

[k] Statistically significant increase for high dose, within historical control , not judged to be treatment-related

[l] Two groups treated with same level of Roundup GBF but one group also treated with vitamin E.

[m] Increases in abnormal metaphases not statistically significant excluding gaps from aberrant cells. Authors conclude positive result based on statistically significant increases in abnormal metaphases including gaps.

[n] Statistically significant increase for high dose at 48 hours, within historical control, but judged to be due to a low control group value and not treatment related.

Table 4. High Dose and Control MN PCE Frequencies for Regulatory Glyphosate and Glyphosate Salt Studies.

Test Material[a]	Sex	Dose	Route	Harvest	Micronucleated PCE per 1000 PCE mean ± std. dev.		Reference
		(mg/kg bw)		(h)	Control	High Dose	
G	M	5000	p.o.	24		1.7±0.6	Jensen (1991c)
				48	1.5± 0.7	1.1±0.4	
				72		0.9±0.7	
	F	5000		24		1.5±0.7	
				48	1.2± 0.3	1.7±0.8	
				72		0.8±0.6	
G	M	5000	p.o.	24	6.7±5.5	8.8±1.8	Suresh (1993b)
	F	5000		24	4.9±2.7	10.4±4.9*	
G	M	5000	p.o.	24	1.6±0.8	2.1±1.6	Fox &Mackay (1996)
				48	1.7±1.3	2.1±1.9	
	F	5000		24	1.4±0.7	2.1±2.5	
				48	0.7±0.6	0.8±0.8	
GK	M	2000	p.o.	24	0.2±0.4	0.9±0.4*	Jones (1999)

				48	0.8±1.0	0.9±1.0	
G	M	562.5	i.p.	24	0.4±0.5	0.4±0.9	Marques (1999)
	F	562.5		24	0.8±0.8	0.6±0.5	
GI	M	3024	i.p.	24	0.6±0.5	0.7±1.0	Gava (2000)
	F	3024			0.4±0.5	0.7±1.0	
G	M	2000	p.o.	24	0.9±0.6	0.9±0.7	Honarvar (2005)
	F	2000		24	0.7±0.8	0.6±0.7	
	M	2000		48		1.5±1.0	
	F	2000		48		1.1±0.9	
G	M	600	i.p.	24	0.6±0.6	1.9±0.7*	Durward (2006)
				48	1.0±1.2	0.9±1.1	
G	M	30	p.o.	24	0.6±0.3	1.4±0.4*	Zoriki Hosomi (2007)
G	M	2000	p.o.	24	0.7±0.7	0.7±0.4	Honarvar (2008)
		2000		48	0.7±0.6	0.8±0.6	
G	M	62.5	i.p.	24	0.0±0.0	0.3±0.7	Costa (2008)
	F	62.5		24	0.0±0.0	0.0±0.0	
G	M (rat)	2000	p.o.	24	0.8±0.6	0.6±0.4	Flugge (2009b)

				48	1.0±0.9	0.8±0.4	
	F (rat)	2000		24	0.9±0.2	0.4±0.4	
				48	1.1±0.7	0.4±0.4	

[a] G, glyphosate technical acid; GK, potassium salt of glyphosate; GI, isopropylamine salt of glyphosate

* Statistically significant increase over control value.

Table 5. Blood Erythrocyte Micronucleus Assays in Non-Mammalian Systems,

Test System	Test Material	Maximum Dose[a]	Result	Comment[b]	Reference
Oreochromis niloticus (Tilapia)	Roundup 69	170 mg/kg i.p. (maximum tolerated)	Equivocal[c]		Coutinho do Nascimento and Grisolia (2000)
Tilapia	Roundup™ formulation	170 mg/kg (abdominal injection)	Positive		Grisolia (2002)
Crasseus auratus (goldfish)	Roundup™ formulation	15 ppm glyphosate in water (2, 4 and 6 days)	Positive		Cavas and Konen (2007)
Prochilodus lineatus (tropical fish)	Roundup™ formulation	10 mg/L **in water** (6, 24 and 96 h)	Negative	NC	Cavalcante et al. (2008)
Caiman eggs/ hatchlings	Roundup® Full II formulation	1750 µg/egg	Positive		Poletta et al. (2009)

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Caiman eggs/ hatchlings	Roundup® Full II formulation	Nest sprayed 3% (3 l/100 l water/ha ~~2X- 30 days apart~~	Positive		Poletta et al. (2011)
O. cordobea (amphibian)	Roundup formulation	100 mg a.i./L	Negative?[d]		Bosch et al. (2011)
R. arenarum (amphibian)		800 mg a.i./L	Equivocal?[e]		
Corydoras paleatus (fish)	Roundup® formulation	6.67 µg/L in water (3.2 µg/L a.e.) (3, 6 and 9 days)	Negative	PC, NC	de Castilhos Ghisi and Cestari (2012)

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[a] a.e., concentration in glyphosate acid equivalents.; a.i. concentration of active ingredient

[b] PC, no concurrent positive control; NC, independent coding of slides for scoring not explicitly indicated for visually scored slides. In some cases coding may have been implied by reference citation.

[c] Statistically significant increase in micronucleated erythrocyte frequency only at mid dose level.

[d] Increase in micronucleated erythrocyte frequency not statistically significant for single group surviving treatment; authors appear to conclude increase may have been treatment related.

[e] Authors appear to conclude increases in micronucleated erythrocytes were treatment related. No statistically significant differences were observed among the experimental groups by analysis of variance. A statistically significant positive correlation between concentration and micronucleated erythrocyte frequency but this analysis apparently omitted the high dose group.

Table 6. DNA Damage Assays of Glyphosate, Glyphosate Salts and GBFs in *In Vitro* and *In Vivo* Mammalian Systems

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro Studies Glyphosate and Glyphosate Salts						
Literature Studies						
Comet	GM38 human fibroblasts	glyphosate (technical)	6.5 mM	Positive	MA, PH, NC	Monroy et al. (2005)
	HT1090 human Fibrosarcoma	glyphosate (technical)	6.5 mM	Positive	MA, PH, NC	Monroy et al. (2005)
SCE	bovine lymphocytes	glyphosate (62% Isopropylamine salt)	1.12 mM (toxic)	Positive (-S9) Equivocal (+S9)	PH, NC	Sivikova and Dianovsky (2006)

Comet	Hep-2 cells	glyphosate (analytical, 96%)	7.5 mM (limited by toxicity)	Positive	MA, PH?, NC	Manas et al. (2009)
Comet	Human lymphocytes	Glyphosate (technical, 98%)	580 µg/mL (toxic) (≈3.43 mM)	Positive (-S9) Positive (+S9)	NC	Mladinic et al. (2009a)
Comet	TR146 human buccal epithelial	Glyphosate (95%)	2000 mg/L (≈11.2 mM)	Positive	MA, PH, NC	Koller et al. (2012)

Regulatory Study

UDS	Primary rat Hepatocyte	Glyphosate (>98%)	111.69 mM	Negative	PH	Rossberger (1994)

In Vitro Studies GBF

Literature Studies

SCE	mouse spleen cells	herbazed formulation (84% glyphosate)	50 mM glyphosate?[b]	Positive	MA, PH, TO, NC	Amer et al. (2006)
Comet	TR146 human buccal epithelial	Roundup™ Ultra Max	200 mg/L glyphosate (≈1.12 mM)	Positive	MA, PH, NC	Koller et al. (2012)

In Vivo Studies GBF

Literature Studies

Bone marrow SCE	Mouse	herbazed formulation (84% glyphosate)	200 mg/kg p.o. glyphosate	Positive	NC	Amer et al. (2006)

[PAGE * MERGEFORMAT]

[a] MA, Mammalian metabolic activation system not used; PH, no indication of pH or osmolality control; TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level; NC, independent coding of slides for scoring not explicitly indicated for visually scored slides.

[b] Calculated from the stated concentration of 5×10^{-5} M glyphosate/mL

Table 7. DNA Damage Assays of Glyphosate, Glyphosate and GBF's In Non-Mammalian Systems

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[b]	Reference
***In Vitro* Studies Glyphosate and Glyphosate Salts**						
Literature Studies						
Comet	Tradescantia flowers and nuclei	Glyphosate (technical, 96%)	0.7 mM	Positive	NC	Alvarez-Moya et al. (2011)
Comet	Oyster sperm	Glyphosate	5 µg/L (≈0.03 µM)	Negative	NC	Akcha et al. (2012)
Regulatory Study						
Rec assay	B. subtilis	Glyphosate 95.68%)	240 µg/disk	Negative		Akanuma (1995a)
In Vitro Studies GBF's						

Literature Studies

SOS[a]	E. coli	Roundup BIO formulation	0.25 µg/sample	Positive		Raipulis (2009)
Sperm Comet	Oyster	Roundup Express®	5 µg/L glyphosate (≈0.03 uM)	Negative	NC	Akcha et al. (2012)

In Vivo Studies GBF's

Literature Studies

Comet	Freshwater mussel larvae	Roundup formulation	5 mg/L glyphosate	Negative	NC	Conners and Black (2004)
Erythrocyte Comet	Crasseus auratus (goldfish)	Roundup formulation	15 ppm glyphosate in water (2, 4 and 6 days)	Positive		Cavas and Konen (2007)

Erythrocyte and gill cell Comet	Prochilodus lineatus (tropical fish)	Roundup™ formulation	10 mg/L in water (6, 24 and 96 h)	Positive		Cavalcante et al. (2008)
Erythrocyte Comet	Caiman eggs /hatchlings	Roundup® Full II formulation	1750 µg/egg	Positive		Poletta et al. (2009)
Erythrocyte Comet	*Anguilla anguilla* (eel)	Roundup™ formulation	36 µg/L glyphosate 1 and 3 days	Positive	NC	Guilherme et al. (2010)
Erythrocyte Comet	Caiman eggs /hatchlings	Roundup® Full II formulation	Nest sprayed 3% (3 l/100 l water/ha ~~2X 30 days apart~~	Positive		Poletta et al. (2011)
Liver and gill cell Comet	*Anguilla anguilla* (eel)	Roundup® Ultra	36 µg/L glyphosate (1 and 3 days)	Positive	NC	Guilherme et al. (2012)

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a SOS response DNA damage assay.

[b] NC, independent coding of slides for scoring not indicated for visually scored slides. In some cases coding may have been implied by reference citation.

Figure Legends

Figure 1. Chemical structure of glyphosate, (N-(phosphonomethyl)glycine, CAS 1071-83-6). (a) neutral form; (b) ionic form.