4. Literature Review of Genotoxicity Publications

The following genotoxicity literature review was conducted by an expert in the field of genotoxicology. Relevant OECD Tier II-like summaries and Klimisch ratings (Klimisch, 1997), as described in introduction of the overall literature review, follow this genotoxicity literature review.

Review of Genotoxicity of Glyphosate and Glyphosate Based Formulations,
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Abbreviations AMPA, aminomethylphosphonic acid ; CB MN, cytokinesis block micronucleus; GBF, glyphosate based formulation; i.p., intraperitoneal ; NCE, normochromatic erythrocyte; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocyte; POEA, polyethoxylated tallow amine, tallowamine ethoxylate; SCE, sister chromatid exchange; SCGE, single cell gel electrophoresis (comet).

Abstract

An earlier review of the toxicity of glyphosate and the original Roundup™ formulation concluded that neither glyphosate nor the formulation pose a risk for the production of heritable/somatic mutations in humans (Williams et al., 2000). This review of subsequent glyphosate genotoxicity publications includes analysis of study methodology and incorporation of all the findings into a weight of evidence for genotoxicity. Two publications provided limited additional support for the conclusion that glyphosate and glyphosate based formulations (GBFs) are not active in the gene mutation assay category. The weight of evidence from *in vitro* and *in vivo* mammalian chromosome effects studies supports the earlier conclusion that glyphosate and GBFs are predominantly negative for this endpoint category. Exceptions are mostly for unusual test systems but there are also some unexplained discordant positive results in mammalian systems. Several reports of positive results for the SCE and comet DNA damage endpoints have been published for glyphosate and GBFs. The data suggest that these DNA damage effects are likely due to cytotoxic effects rather than DNA reactivity. This weight of evidence review concludes that there is no significant *in vivo* genotoxicity and mutagenicity potential of glyphosate or GBFs that would be expected under normal exposure scenarios.

1. Introduction

Glyphosate is the active ingredient in very widely used herbicide formulations in crop production, industrial turf, ornamental plants, forestry, roadsides, home lawns and gardens. Accordingly, the toxicity of glyphosate and its formulated products has been extensively studied. An earlier thorough review of glyphosate and glyphosate formulation safety and risk assessment included descriptions and analyses of genetic toxicology studies of glyphosate, the original Roundup™ formulation and other glyphosate based formulations (GBFs) (Williams et al., 2000). Subsequently, a fairly large number of genetic toxicology studies of glyphosate and GBFs have been published. These studies include a wide variety of test systems and endpoints. The number and diversity of the studies warrant careful examination and integration of their findings with the previous results to produce an updated assessment of the overall genotoxicity profile of glyphosate and GBFs.

2. General Review and Analysis Considerations

The published studies for review consideration were identified by literature searches for published reports containing references to glyphosate or GBFs that also contained searchable terms which indicated that genotoxicity studies were performed. Literature search utilized Chemical Abstracts (provided by Chemical Abstracts Service, a division of the American Chemical Society) and Web of Knowledge (Thompson Reuters), using the following modules: Web of Science℠, BIOSIS Previews®, MEDLINE®, and CAB Abstracts® (CABI) abstracting services. Search criteria were as follows (glyphosate acid and the various salts): glyphosat* OR glifosat* OR glyfosat* OR 1071-83-6 OR 38641-94-0 OR 70901-12-1 OR 39600-42-5 OR 69200-57-3 OR 34494-04-7 OR 114370-14-8 OR 40465-66-5 OR 69254-40-6 OR (aminomethyl w phosphonic*) OR 1066-51-9. Each identified publication was evaluated to verify that it contained original results of one or more genotoxicity studies on glyphosate or GBFs. Emphasis was placed on publications in peer-reviewed journals and 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 developed 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 categories of studies or 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 Cooperation and Development (OECD) are a pre-eminent source of internationally agreed and expert guidelines. Other regulatory international and national regulatory genetic toxicology testing guidance are usually concordant with the OECD guidelines. Table 1 presents 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 it does serve to provide one means of characterization of the various published studies. Some of the criteria are rarely met in scientific publications. 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 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 in demonstrating 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) 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 include a description of coding or "blind" scoring in the methodology would appear to have a deficiency either in the methodology or the description of the methodology used. 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.

Test materials, as described in the publications, were reviewed by industry experts to identify any publicly available and useful information on composition for the reported formulations to assist in interpreting the relevance of findings to glyphosate and/or formulation components. 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 what may be any GBF that contains additional components such as surfactants. Published results from studies with different formulations have sometimes been incorrectly or inappropriately attributed to the active ingredient. The original Roundup formulation (containing 41% isopropyl amine 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 than the active ingredient. The fact that test materials identified as Roundup formulations may actually have different compositions should be considered when comparing results of different studies. A major consideration, especially for DNA damage endpoints and for *in vitro* mammalian cell assays, is an assessment of whether observed effects might be due to toxicity or extreme culture conditions rather than indicating DNA-reactive mediated processes (Dearfield et al., 2011; Muller and Kasper, 2000; Scott et al., 1991; Thybaud et al., 2007b; Thybaud et al., 2007a). Relevant considerations include control of medium pH and osmolality for *in vitro* mammalian cell studies and whether effects are observed only at cytotoxic doses or in association with severe toxicity to the test system. Other important generic considerations in evaluating experimental results of each published study are evidence of experimental reproducibility and whether a biologically plausible dose response has been demonstrated.

Table 1. Genetic Toxicology Test Guideline Criteria

Area	Guidance	Reference
All studies	Test material purity and stability should be reported	OECD 471 (1997) OECD 473 (1997)
	Concurrent negative and positive controls should be included with each assay	
Assays with visual scoring	All slides should be independently coded before analysis (i.e. scored without knowledge of the treatment or control group)	OECD 473 (1997) OECD 479 (1986)
In vitro mammalian cell assays	Assay should be usually be conducted in the presence and absence of an appropriate exogenous metabolic activation system	OECD 473 (1997)
	Cytotoxicity should be determined in the main experiment	
	At least three analyzable concentrations should be used	
	Maximum dose determined by toxicity or 5 µg/ml, 5 mg/ml or 10 mM for soluble non-toxic test materials	
	Individual culture data should be provided	
In vivo mammalian assays	Five analyzable animals per group. Single sex may be used if there are no substantial difference in toxicity between sexes	OECD 475 (1997) OECD 474 (1997)
	Limit dose for non-toxic substances of 2000 mg/kg for treatments up to 14 days and 1000 mg/kg for treatments longer than 14 days	
In vitro chromosome aberration	Treatment for 3-6 hours in one experiment and harvest at 1.5 cell cycles. If negative a second experiment with continuous treatment for 1.5 cell cycles	OECD 473 (1997)
	Scoring of at least 200 metaphases ideally divided between duplicate cultures	
In vitro sister chromatid exchange	Treatment for 1-2 hours up to two cell cycles with harvest after two cell cycles in the presence of bromodeoxyuridine	OECD 479 (1986)
	Scoring of 25 metaphases per culture (50 per treatment group)	
In vitro micronucleus	Most active agents detected by treatment for 3-6 hours with harvest at 1.5-2 cell cycles after treatment. An extended treatment for 1.5-2 cycles in the absence of metabolic activation is also used	OECD 487 (2010)
	Scoring of at least 2000 binucleated cells or cells for micronuclei for each treatment or control group	
In vivo bone marrow chromosome aberration	Single treatment with first harvest at 1.5 cell cycles after treatment and second harvest 24 hour later or single harvest 1.5 cycles after last treatment for multiple daily treatments	OECD 475 (1997)
	Three dose levels usually recommended except when limit dose produces no toxicity	
	Concurrent measures of animal toxicity and toxicity to target cells	
	At least 100 cells analyzed per animal	
	Individual animal data should be reported	
In vivo erythrocyte micronucleus	Three dose levels for first sampling time	OECD 474 (1997)
	Treatment once with at least 2 harvests usually at 24 and 48 h after treatment or one harvest 18-24 h after final treatment if two or more daily treatments are used	
	Scoring of 2000 immature erythrocytes per animal or 2000 mature erythrocytes for treatments of 4 weeks or longer	

Table 2 presents a summary of genotoxicity test results for glyphosate and GBFs published subsequent to Williams et al. (2000). Test results are organized by the major genotoxicity assay categories of gene mutation, chromosome effects and DNA damage and other endpoints. Major features presented for each publication are the assay endpoint, the test system, the test material, the maximum dose tested and comments relevant to the reported conduct and results of the assay. For brevity, earlier reviewed individual publications of genotoxicity study results are referred to by citation of (Williams et al., 2000) rather than the original references reviewed in (Williams et al., 2000).

Table 2. Genetic Toxicology Studies of Glyphosate and Glyphosate Formulations Published On or After 2000

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro Gene Mutation						
Point mutation	Ames strains	Perzocyd 10 SL formulation	2 µg/plate (toxic)	Negative	TA1535 not used	Chruscielska et al., 2000
Wing spot test	Drosophila	glyphosate (96%)	10 mM in larval stage	Negative/ inconclusive[c]	Negative or inconclusive in crosses not sensitive to recombination events	Kaya et al., 2000
In Vitro Chromosome Effects—Mammalian Systems						
Cytokinesis block micronucleus	Bovine lymphocytes	Glyphosate formulation (62% glyphosate Monsanto source)	560 µM 48 h –S9	Positive?	PH, MA, SC, TO	Piesova, 2004
Cytokinesis block micronucleus	Bovine lymphocytes	Glyphosate formulation (62% glyphosate Monsanto source)	560 µM 48 h –S9 2 h –S9 2 h +S9	Positive? Negative Negative	PH, SC, TO	Piesova, 2005
Chromosome aberration	Mouse spleen cells	herbazed formulation	50 µM?	Positive	Concentrations used not clear. PH, MA, SC, TO, RE	Amer et al., 2006
Chromosome aberration	Bovine lymphocytes	Glyphosate formulation (62% glyphosate) Monsanto source	1.12 mM (toxic) (24 h)	Negative	Chromosome 1 FISH analysis. PH, MA, PC, SC, TO, RE	Holeckova, 2006
Chromosome aberration	Bovine lymphocytes	Glyphosate formulation (62% glyphosate) Monsanto source	1.12 mM (toxic) (24 h)	Negative	PH, MA, SC, RE	Sivikova and Dianovsky, 2006
Chromosome aberration	Human lymphocytes	Glyphosate (96%)	6 mM (not toxic)	Negative	MA, IC, RE	Manas et al., 2009b
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009a
Cytokinesis block micronucleus	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/mL (toxic) (est. 3.43 mM)	Negative (-S9) Positive (+S9)	SC, RE	Mladinic et al., 2009b

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro Chromosome Effects— Non Mammalian Systems						
Chromosome aberration	Onion root tip meristem	Roundup formulation (Bulgaria)	1% active ingredient (estimated 4.4-5.9 mM)	Negative	TO, IC, RE	Dimitrov et al., 2006
Micronucleus	Onion root tip meristem	Roundup formulation (Bulgaria)	1% active ingredient (estimated 4.4-5.9 mM)	Negative	TO, RE	Dimitrov et al., 2006
In Vivo Chromosome Effects—Mammalian Systems						
Bone marrow erythrocyte micronucleus	Mouse	Glyphosate	300 mg/kg i.p. Perzocyd 10 SL formulation	Negative Negative	DL, TO, SC, IM, RE DL, TO, SC, IM, RE	Chruscielska et al., 2000
Bone marrow erythrocyte micronucleus	Mouse	Roundup 69 formulation	2 x 200 mg/kg i.p.	Negative	TO, SC, IE, RE	Coutinho do Nascimento and Grisolia, 2000
Bone marrow erythrocyte micronucleus	Mouse	Roundup™ formulation (Monsanto)	2 x 200 mg/kg i.p.	Negative	TO, SC, IE, RE	Grisolia, 2002
Bone marrow Chromosome aberration	Rabbit	Roundup™ formulation	750 ppm in drinking water	Positive?	DL, PC, TO, SC, IC	Helal and Moussa, 2005
Bone marrow Chromosome aberration	Mouse	Herbazed formulation (84% glyphosate)	50 mg/kg i.p. (1,3, 5 days) 100 mg/kg oral (1,7, 14, and 21 days)	Negative Positive	TO, SC, RE	Amer et al., 2006
Spermatocyte Chromosome aberration	Mouse	Herbazed formulation (84% glyphosate)	50 mg/kg i.p. (1,3, 5 days) 100 mg/kg oral (1,7, 14, and 21 days)	Negative Positive	TO, SC, RE	Amer et al., 2006
Bone marrow Chromosome aberration	Mouse	Roundup formulation (Bulgaria)	1080 mg/kg p.o. (1/2 LD_{50})	Negative	DL, TO, IC, RE	Dimitrov et al., 2006
Bone marrow erythrocyte micronucleus	Mouse	Analytical glyphosate (96%)	2 x 200 mg/kg i.p.	Positive	Erythrocytes scored? TO, SC, IC, RE	Manas et al., 2009b
Bone marrow Chromosome aberration	Mouse	Roundup™ formulation (Monsanto)	50 mg/kg i.p.	Positive	DL, SC, IC, RE	Prasad et al., 2009

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vivo Chromosome Effects—Non-Mammalian Systems						
Erythrocyte micronucleus	Oreochromis niloticus (Tilapia)	Roundup 69	170 mg/kg i.p. (maximum tolerated)	Negative?[c]	TO, RE	Coutinho do Nascimento and Grisolia, 2000
Wing spot test	Drosophila	Glyphosate (96%)	10 mM in larval stage	Positive/inconclusive[b]		Kaya et al., 2000
Erythrocyte micronucleus	Tilapia	Roundup™ formulation (Monsanto)	170 mg/kg (abdominal injection)	Positive	TO, RE	Grisolia, 2002
Erythrocyte micronucleus	*Crasseus auratus* (goldfish)	Roundup formulation	15 ppm glyphosate in water (2, 4 and 6 days)	Positive	TO, IE, RE	Cavas and Konen, 2007
	Prochilodus lineatus (tropical fish)	Roundup™ formulation (75% of 96 h LC50)	10 mg/l (6, 12 and 24 h) in water	Negative	DL, TO, SC, RE	Cavalcante et al., 2008
Erythrocyte micronucleus	Caiman eggs	Roundup® Full II formulation	1750 ug/egg	Positive	RE	Poetta et al., 2009
Erythrocyte micronucleus	Caiman eggs	Roundup® Full II formulation	Sprayed 2x with 100 litres of 3%/ha 30 days apart	Positive	DL, TO, RE	Poetta et al., 2010
In Vitro DNA Damage Mammalian Systems						
Alkaline SCGE	GM38 human fibroblasts and HT1090 human fibrosarcoma	Glyphosate (technical grade)	6.5 mM	Positive	MA, PH, TO, SC, RE	Monroy et al., 2005
Sister chromatid exchange	mouse spleen cells	herbazed formulation	50 µM?	Positive	Concentrations used not clear MA, PH, TO, SC, RE	Amer et al., 2006
Sister chromatid exchange	bovine lymphocytes	Glyphosate formulation (62% glyphosate, Monsanto)	1.12 mM (toxic)	Positive	PH, SC, RE	Sivikova and Dianovsky, 2006
Alkaline single cell gel electrophoresis (SCGE, comet)	Hep-2 cells	Glyphosate (analytical, 96%)	7.5 mM (limited by toxicity)	Positive	MA, PH, RE	Manas et al., 2009b
Alkaline SCGE	Human lymphocytes	Glyphosate (technical, 96%)	580 µg/ml (toxic) (est. 3.43 mM)	Positive (-S9) Positive (+S9)		Mladinic et al., 2009a

Endpoint	Test System	Test Material	Maximum Dose	Result	Comment[a]	Reference
In Vitro DNA Damage Non-Mammalian Systems						
SOS	*E. coli*	Roundup BIO formulation	2.5 ug/sample	Positive		Raipulis J, 2009
Alkaline SCGE	Tradescantia flowers and nuclei	Glyphosate(technical, 96%)	700 µM	Positive	PH, SC	Alvarez-Moya et al., 2011
Bone marrow SCE	Mouse	herbazed formulation (84% glyphosate)	200 mg/kg p.o.	Positive	TO, SC, RE	Amer et al., 2006
Sperm abnormality	Mouse	herbazed formulation (84% glyphosate)	200 mg/kg p.o. (5 days)	Positive	TO, SC, RE	Amer et al., 2006
In Vivo DNA Damage Non-Mammalian Systems						
Erythrocyte Alkaline SCGE	Freshwater mussel larvae	Roundup formulation	5 mg/liter	Negative	TO, SC	Conners and Black, 2004
Erythrocyte alkaline SCGE	*Crasseus auratus* (goldfish)	Roundup formulation	15 ppm glyphosate in water (2, 4 and 6 days)	Positive	TO, RE	Cavas and Konen, 2007
Erythrocyte and gill cell alkaline SCGE	*Prochilodus lineatus* (tropical fish)	Roundup™ formulation (75% of 96 h LC50)	10 mg/l (6, 12 and 24 h) in water	Positive	DL, TO, RE	Cavalcante et al., 2008
Erythrocyte alkaline SCGE	Caiman eggs/hatchlings	Roundup® Full II formulation	1750 µg/egg	Positive	RE	Poletta et al., 2009
Erythrocyte alkaline SCGE	European eel	Roundup formulation	166 µg/liter	Positive	DL, SC, RE	Guilherme et al., 2010
Erythrocyte alkaline SCGE	Caiman eggs/hatchlings	Roundup® Full II formulation	Sprayed 2x with 100 l of 3%/ha 30 days apart	Positive	DL, RE	Poletta et al., 2010

[a] MA, Mammalian metabolic activation system not used and short exposure not used;
PH, no indication of pH or osmolality control;
DL, less than three dose levels used; PC, no concurrent positive control;
TO, no concurrent measurement of toxicity reported or toxicity not observed for highest dose level;
SC, independent coding of slides for scoring not indicated for visually scored slides;
IC, less than 200 cells scored per treatment or less than 100 metaphases scored per animal for chromosome aberrations.;
IE, less than 2000 erythrocytes scored per animal;
RE, results not reported separately for replicate cultures or individual animals;.

[b] Positive for small wing spots only in one cross. Negative or inconclusive for all spot categories for three other crosses.

[c] Statistically significant increase in micronucleated PCE frequency only at mid dose level but overall result judged negative.

3. Structure Activity Analysis

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

4. Gene Mutation

As reviewed by Williams et al., (2000), most gene mutation studies for glyphosate and GBFs were negative. Gene mutation assays included numerous Ames/*Salmonella* and *E. coli* WP2 bacterial reversion assays, *Drosophila* sex-linked recessive lethal assays and a CHO/HGPRT *in vitro* mammalian cell assay. Of fifteen gene mutation assays reported, there were only two positive observations. A reported positive Ames/*Salmonella* result for Roundup formulation was not replicated in numerous other studies. There was 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).

Subsequent to the Williams et al. (2000) review only two gene mutation studies have been reported (Table 2). One negative Ames/*Salmonella* assay result was reported for a GBF of undefined composition, Percozyd 10 SL (Chruscielska et al., 2000). Although this result is consistent with a large number of negative Ames/*Salmonella* results for glyphosate and GBFs, 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 revertant/plate data. 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., 2004). Small increases in small wing spot frequencies were observed in one of four crosses of larvae treated with up to 10 mM glyphosate. 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.

These gene-mutation publications add very limited data to the weight of evidence conclusion that glyphosate and GBFs do not pose significant risk for gene mutation.

5. Chromosome Effects

Assays to detect chromosome effects such as structural chromosome aberrations and micronucleus incidence constitute a second major genotoxicity endpoint category. A large number of publications with chromosome effects endpoints have been reported since the Williams et al. (2000) review. These are described in Table 2 and are separated into various test system categories which include *in vitro* cultured mammalian cell assays, *in vitro* tests in non-mammalian systems, *in vivo* mammalian assays and *in vivo* assays in non-mammalian systems. A *Drosophila* wing spot test (discussed previously) is also included in this category because results are relevant to somatic recombination.

5.1 In vitro Chromosome Effects

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 approximately 2-3 mM (calculated from reported mg/ml) in the absence and presence of an exogenous mammalian activation system. 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 earlier review noted several other unusual features about the positive result studies including an unusual exposure protocol and discordant positive results for another chemical found negative in other laboratories.

As indicated in Table 2 both positive and negative results have been reported for glyphosate and GBFs in the nine *in vitro* chromosome effects assays published after the Williams et al. (2000) review. It is noteworthy that many of these studies have various deficiencies in conduct or reporting compared to internationally accepted guidelines for conduct of *in vitro* chromosome aberration or micronucleus studies (see Table 1). Perhaps the most significant deficiency was that coding and scoring of slides without knowledge of the treatment or control group was not indicated in seven of nine publications. This could be a deficiency in conducting the studies or perhaps a deficiency in describing methodology in the publications. Other common deficiencies included failure to indicate control of exposure medium pH, no use of exogenous metabolic activation and no reporting of concurrent measures of toxicity.

5.1.2 Results for glyphosate active ingredient

Three publications reported testing of technical glyphosate for micronucleus or chromosome aberration endpoints in cultured human lymphocytes (Manas et al., 2009b; Mladinic et al., 2009a; Mladinic et al., 2009b). Negative results for the micronucleus or 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 in these studies.

Two publications by one author reported cytokinesis block micronucleus results for cultured bovine lymphocytes treated with what was reported as 62% by weight isopropyl amine salt of glyphosate from a Monsanto Belgium source (Piesova, 2004; Piesova, 2005). 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 GBF. In one publication no statistically significant increases in binucleated cell micronucleus frequency were observed with 24 hours of treatment (Piesova, 2004). For 48 hours of treatment a statistically significant increase in micronucleus frequency was observed in one donor at 280 µM but not at 560 µM and in a second donor at 560 µM but not 280 µM. The second publication reported negative results for the cytokinesis block micronucleus assay in bovine lymphocytes incubated with glyphosate formulation up to 560 µM for two hours in the absence and presence of a mammalian metabolic activation system (Piesova, 2005). This publication also reported positive results for 48 hours of treatment without S9. Curiously, in this second publication the same inconsistent dose response pattern was observed in which a statistically significant increase in micronucleus frequency was observed in one donor at 280 µM but not at 560 µM and in a second donor at 560 µM but not 280 µM. The lack of a consistent dose response pattern between donors suggests that the results with 48 hours of treatment are questionably positive.

Two other publications found negative results for the chromosome aberration endpoint in cultured bovine lymphocytes treated with what appears to be the same test material of 62% by weight isopropylamine salt of glyphosate from a Monsanto Belgium source, (Holeckova, 2006; Sivikova and Dianovsky, 2006). Both the studies used a maximum concentration of 1.12 mM which was reported to cause a decrease in mitotic inhibition of >50%. These two studies have several limitations including that an exogenous mammalian metabolic activation system was not used for chromosome aberration and scoring was not reported to be on coded slides. In addition, Holeckova (2006) only examined effects detectable by staining of chromosome 1 and did not report positive control results (Holeckova, 2006). Despite these limitations and the variable donor results, the results from these two studies are generally consistent with a lack of chromosome aberration effects of the isopropylamine salt of glyphosate on *in vitro* cultured mammalian cells in several experiments using high, toxic dose levels and exposures of 2-24 hours in the absence of S9.

One laboratory reported increases in cytokinesis-blocked micronucleus frequency in cultured human lymphocytes exposed to glyphosate for 4 hours in the presence of an exogenous human liver metabolic activation system (S9) in two publications (Mladinic et al., 2009a; Mladinic et al., 2009b). 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). Increased proportions of centromere- and DAPI-positive micronuclei were observed for the high dose with S9 suggesting that the induced micronuclei were derived from chromosomes rather than chromosome fragments. 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 were uniquely or preferentially observed in the presence of S9 and at the highest dose level tested (Mladinic et al., 2009a). Also, the negative control level of such endpoints as necrosis and alkaline SCGE tail moment was 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). These observations suggest that the observations of S9 mediated effects by Mladinic et al. are not likely to be due to *in vivo* relevant metabolites. It is possible that such effects might be generated by *in vitro* S9-mediated processes that are not relevant to *in vivo* processes such as genotoxic effects of low pH observed in the presence of S9 in *in vitro* assays (Cifone et al., 1987). The preponderance of *in vitro* genotoxicity studies conducted with exogenous mammalian metabolic activation systems has been negative, including a previously reviewed chromosome aberration study in human lymphocytes conducted up to a similar dose

level (Williams et al., 2000) and a bovine lymphocyte cytokinesis block micronucleus study (Piesova, 2005). Overall these results suggest the possibility of a weak aneugenic rather than clastogenic (chromosome breaking) effect occurring in the presence of S9 at high dose levels of glyphosate. The pattern of activity as well as the failure to observe activity in several other *in vitro* genotoxicity assays conducted with S9 suggests that the activity observed in the Mladinic et al. studies does not have a significant weight of evidence for *in vitro* genotoxicity and is not likely to be relevant to *in vivo* genotoxicity.

The recently published results for mammalian *in vitro* chromosome aberration and micronucleus assays demonstrate a weight of evidence that technical glyphosate and glyphosate salt concentrates are negative for these endpoints in cultured mammalian cells in the absence of an exogenous mammalian metabolic activation system. Five publications from four laboratories report negative *in vitro* mammalian cell chromosome or micronucleus results in the absence of exogenous activation while three publications from two laboratories report positive results. 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 aberrant.

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) have no substantial reproducibility verification from other laboratories in the recent *in vitro* chromosome effects studies considered in this review because most of the studies performed by other laboratories (Table 2) did not employ an exogenous mammalian activation system. These results are 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). The numerous consistent negative results for glyphosate and GBFs in gene mutation studies which employed exogenous mammalian metabolic activation and careful examination of the data suggests that the positive results indicate a possible threshold aneugenic effect associated with cytotoxicity rather than a DNA-reactive mechanism resulting in chromosome breakage. Thus, the weight evidence for the *in vitro* chromosome effect assays indicates a lack of DNA-reactive clastogenic chromosome effects.

5.1.3 Results for GBFs

Amer et al. (2006) reported positive *in vitro* chromosome aberration effects in mouse spleen cells for a formulation described as herbazed, 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, lacking description of the glyphosate salt form and inert ingredients. The glyphosate concentrations used in the study are not clear because there are different descriptions of the concentration units (M or M glyphosate/ml medium) in the publication. Thus, the maximum concentration might have been 5 x 10^{-5} M (50 µM) or 5 x 10^{-5} M glyphosate/ml medium (50 mM). The former concentration, which was reported as toxic, would indicate effects at concentrations well below those typically found toxic for GBFs in cultured mammalian cells. The latter level of 50 mM would be well in excess of the limit level of 10 mM recommended in OECD guidelines (OECD473, 1997). In addition to a question about the concentration 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, no reported concurrent toxicity measurements 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 or reliability with respect to glyphosate or GBFs.

In addition to *in vitro* mammalian cell studies there is also a report of negative results for the chromosome aberration and micronucleus endpoints in onion root tips incubated with a Roundup formulation (Dimitrov et al., 2006). The maximum exposure concentration (stated as 1% active ingredient) is estimated to be on the order of 4-6 mM. This study did not employ an exogenous mammalian metabolic activation system; however, it does provide evidence for a lack of chromosome effects for glyphosate and a GBF in a non-mammalian *in vitro* system. The result agrees with earlier reported negative onion root tip chromosome aberration results for glyphosate but is discordant with earlier reported positive results for a Roundup GBF in this system (Williams et al., 2000).

5.2 In vivo Chromosome Effects—Mammalian Systems

The Williams et al. (2000) glyphosate toxicity review presented results from *in vivo* mammalian chromosome effect assays. Results from several mouse bone marrow erythrocyte micronucleus studies of glyphosate and GBFs (e.g. Roundup, Rodeo and Direct) 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 and maximum doses for many of the studies were toxic or appropriately close to LD50 values. 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/day. There was one published report of a weak positive mouse bone marrow micronucleus response observed for glyphosate and Roundup 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 glyphosate and GBFs 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.

As indicated in Table 2, there are numerous subsequent publications of *in vivo* mammalian chromosome effects assays. With one exception, all of the *in vivo* mammalian studies were conducted in the mouse using either the bone marrow chromosome aberration or micronucleus endpoints. 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 the bone marrow) are not reported, coding of slides for scoring is not reported, individual animal data are not reported and fewer than recommended cells or metaphases per animal were scored. Other limitations encountered include use of only a single or two dose levels rather than three dose levels.

5.2.1 Results for glyphosate active ingredient

Two publications reported results for glyphosate in the mouse bone marrow erythrocyte micronucleus assay. Negative results were reported in one study which used a dose of 300 mg/kg 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, no reporting of toxicity other than PCE/NCE ratio, no reported coding of slides for scoring and scoring of 1000 PCE's per animal (scoring of 2000 PCE's per animal is recommended by OECD guidelines). A second publication reported positive results for glyphosate administered at 50, 100 and 200 mg/kg via i.p. injections repeated at 24 hours apart with sacrifice 24 hours after the second dose (Manas et al., 2009b). A statistically significant increase in micronucleated erythrocytes was observed in the high dose group. This study had limitations comparable to the negative study. A more significant potential difficulty 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 total 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 (OECD474, 1997).

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 (OECD474, 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 rather than just polychromatic erythrocytes raises a significant methodological question for the reported positive study.

5.2.2 Results for GBFs

There are several publications reporting *in vivo* mammalian bone marrow chromosome aberration and micronucleus endpoint results for Roundup GBFs. Three publications report negative results for Roundup

branded GBF in mouse chromosome aberration or micronucleus assays. Negative results were reported for two different Roundup branded GBFs administered at 2 x 200 mg/kg i.p. in mouse bone marrow erythrocyte micronucleus assays (Coutinho do Nascimento and Grisolia, 2000; Grisolia, 2002). The second study did not report coding of slides for scoring. Another publication reported negative results in mouse bone marrow studies for both the chromosome aberration and erythrocyte micronucleus endpoints (Dimitrov et al., 2006) using a dose of 1080 mg/kg administered orally (p.o.). In contrast, one publication reported positive results for Roundup GBF in mouse bone marrow for the chromosome aberration and erythrocyte micronucleus endpoints using a single maximum dose of 50 mg/kg i.p. (Prasad et al., 2009). Both the positive results and the magnitude of the increases in the chromosome aberration and micronucleus endpoint reported in this study are remarkably discordant with other reported results for Roundup and other GBFs in mouse bone marrow chromosome aberration and erythrocyte studies in a number of laboratories and publications (Table 2 and Williams et al., 2000). The reasons for this discordance are not clear. One unusual feature of the positive study is that the Roundup GBF was administered in dimethylsulfoxide. This is an unusual vehicle to use in *in vivo* genotoxicity studies, particularly for glyphosate which is water soluble and especially so in a formulated product. A published toxicity study found that use of a dimethylsulfoxide/olive oil vehicle by the i.p. route produced dramatically enhanced toxicity of glyphosate formulation or the formulation without glyphosate compared to saline vehicle and that the enhanced toxicity observed with this vehicle was not observed when the oral route was used (Heydens et al., 2008). These observations suggest that use of DMSO as a vehicle for administration of formulation components by the i.p. route might produce unusual toxic effects that are not relevant to normally encountered exposures. Regardless of the reasons for the discordant positive results it is clear that a large preponderance of evidence indicates that GBFs are typically negative in mouse bone marrow chromosome aberration and erythrocyte assays.

One publication reported positive results for bone marrow chromosome aberration in rabbits administered Roundup GBF in drinking water at 750 ppm for 60 days (Helal and Moussa, 2005). This study is relatively unique in terms of species and route of administration. The results do 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 may be attributable to dehydration (Saunders, 2005). This study had further limitations including the use of only a single dose level and not coding slides for scoring. Examination of the chromosome aberration scoring results showed that large increases for the treated group 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 recommended for inclusion in total aberration frequency and centromeric attenuation is not included in ordinary structural aberrations (OECD475, 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 GBFs. 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 LD50 as determined experimentally by the authors. This study had several limitations including use of less than three dose levels and no reported coding of slides for scoring. Positive results were reported for another uncharacterized GBF, herbazed, in mouse bone marrow and spermatocyte chromosome aberration studies (Amer et al., 2006). No statistically significant increases in aberrant cells were observed in bone marrow cells for i.p. treatment of 50 mg/kg for 1, 3 or 5 days or in spermatocytes for 1 or 3 days treatment. Statistically significant increases in frequency of spermatocytes with aberrations were reported for 5 days of treatment with 50 mg/kg (i.p.). Oral treatment of 50 mg/kg and 100 mg/kg were reported to produce increases in aberrant cell frequency in bone marrow cells after extended treatments (14 and 21 days) but not after shorter 1 and 7 day treatments. Similarly, significant increases in aberrant cell frequencies of spermatocytes were reported at 14 and 21 days of 50 mg/kg oral treatment (negative for 1 and 7 days treatment) and at 7, 14 and 21 days of 100 mg/kg treatment (negative for 1 day treatment). Although not a genotoxic endpoint per se, it should be noted that statistically significant increases in frequency of sperm with abnormal morphology were also observed in mice treated with 100 and 200 mg/kg p.o. for 5 days. The positive results for the uncharacterized herbazed GBF were only observed after extended oral treatments (bone marrow and spermatocytes) and extended i.p. treatments (spermatocytes). 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) provides direct evidence that 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.

In vivo mammalian assays for chromosome effects are an important category for characterizing genotoxicity that complements the gene mutation category. While some positive results have been reported the preponderance of evidence and published results are negative for glyphosate and GBFs.

5.3 In vivo Chromosome Effects—Non-Mammalian Systems

The Williams et al. (2000) review reported a few *in vivo* plant assays for chromosome effects in non-mammalian systems. These included negative results for glyphosate and positive results for Roundup GBFs for chromosome aberrations in an onion root tip assay and negative results for glyphosate with the micronucleus endpoint in a *Vicia faba* root tip assay.

Subsequent to the earlier review a number of publications reported results for erythrocyte micronucleus assays conducted on GBFs in several non-mammalian fish and reptile species with discordant results. One publication reported apparently negative 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 an increase in micronucleated erythrocyte frequency at the mid-dose level this was not observed at the high dose level and considerable variability in frequencies in different groups was noted. Negative results were also reported in another fish species (*Prochilodus lineatus*) exposed to 10 mg/liter Roundup branded GBF for 6, 24 and 96 hours (Cavalcante et al., 2008). This concentration was reported to be 96% of a 96 hour LC50. Positive results were reported for the erythrocyte micronucleus assay conducted in the fish *Tilapia rendalii* exposed to 170 mg/kg i.p. of another Roundup 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 was statistically significantly elevated for each of the 7 test materials, including many instances where the statistically significant increases were not consistent with a biologically plausible dose response. 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 of a Roundup GBF for 2 to 6 days (Cavas and Konen, 2007). The reasons for the discordant results are not clear for these fish erythrocyte micronucleus assays of Roundup GBFs. Although different species and GBF's were used in the different studies there were pairs of studies with positive and negative results that used similar treatment conditions (170 mg/kg i.p. or 10-15 mg/liter in water).

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 are evaluated in embryos with only a few days between treatment and the erythrocyte micronucleus endpoint (Wolf et al., 2008). In the reported caiman egg assay there was presumably a single topical exposure followed by an egg incubation period of about 10 weeks before hatching. Biological plausibility raises questions whether genotoxic events *in ovo* can produce elevated micronucleated erythrocyte frequencies detectable after 10 weeks, given the number of cell divisions occurring in development of a hatchling.

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 experiment 2, twelve months later, with the same dosing regimen except the second application at incubation day 35 included cotreatment 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 from control group animals 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 rather than from genotoxic effects induced in the embryos.

One published study reported a weak positive result in a *Drosophila* wing spot assay (Kaya et al., 2004). Statistically significant positive increases were 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.

The above *in vivo* chromosome effect assays in non-mammalian systems give discordant results for reasons that aren't precisely defined. Typically these results would be given lower weight than mammalian systems in being predictive of mammalian effects, especially since there is little or practically no assay 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.

6. DNA Damage and Other Endpoints

A number of studies of glyphosate and GBFs have been published since 2000 which used various DNA damage endpoints in a variety of *in vitro* and *in vivo* systems. The DNA damage category includes endpoints such as sister chromatid exchange and DNA repair response in bacteria, but the most common DNA damage endpoint encountered was the alkaline single cell gel electrophoresis endpoint (alkaline SCGE) also commonly referred to as the "comet" assay. The alkaline SCGE endpoint has been applied to both *in vitro* and *in vivo* test systems.

In addition to DNA damage there are a few reports of other types of studies which can be associated with genotoxic effects even though the endpoints are not specific indicators of genotoxicity per se. These include sperm morphology and carcinogenicity studies.

6.1 In vitro DNA Damage Studies

Some positive results for glyphosate or GBFs in the 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. Additional *in vitro* DNA damage endpoint results described in the earlier review included negative results for glyphosate in the *B. subtilis* rec-assay and in the primary hepatocyte rat hepatocyte unscheduled DNA synthesis assay.

There are two subsequent publications using *in vitro* cultured mammalian cells and the SCE endpoint. Positive SCE results were reported for the uncharacterized herbazed GBF in mouse spleen cells (Amer et al., 2006). The dose response pattern for SCE response in this study was similar to the response for chromosome aberrations in this publication. Limitations of this study are in common to those described above for the chromosome aberration endpoint portion of the study; no indication that pH of treatment solutions was controlled, no use of a mammalian metabolic activation system, no reported concurrent toxicity measurements and no reported use of coded slides for scoring. Positive SCE results were also reported for cultured bovine lymphocytes treated with up to 1.12 mM glyphosate for 24 and 48 hours without exogenous mammalian metabolic activation (Sivikova and Dianovsky, 2006). The highest dose of 1.12 mM significantly delayed cell cycle progression with 48 hour treatment. These same concentrations for 24 h exposures did not induce statistically significant increases in chromosome aberrations which provides a clear example of a differential response of the SCE endpoint (Sivikova and Dianovsky, 2006). This is an important consideration in these publications, as chromosome effects are considered more relevant to genotoxicity than DNA damage.

Positive results for glyphosate are reported for the alkaline SCGE endpoint in three publications. Positive SCGE results were observed for two mammalian cell lines exposed to glyphosate for 4 hours at concentrations of 4.5-6.5 mM (GM39 cells) and 4.75-6.5 mM (HT1080 cells) (Monroy et al., 2005).

These concentrations are close to the upper limit dose of 10 mM generally recommended for *in vitro* mammalian cell assays and control of medium pH is not indicated. Characterization of nuclear damage was done by visual scoring without coding of slides being indicated. Positive alkaline SCGE results were also reported in Hep-2 cells exposed for 4 hours to 3.5-7.5 mM glyphosate (Manas et al., 2009b). Higher concentrations of glyphosate were reported to result in viability of <80% as determined by dye exclusion. As noted for the preceding publication, the concentrations employed were reasonably close to a limit dose of 10 mM and control of medium pH was not reported. This publication reported negative results for the chromosome aberration endpoint in cultured human lymphocytes exposed to up to 6 mM glyphosate for 48 hours and it should be noted that in this case an appropriate control of medium pH was reported for this human lymphocyte experiment. Positive alkaline SCGE results have also been reported for cultured human lymphocytes exposed to glyphosate at concentrations up to 580 µg/ml (estimated 3.4 mM) for 4 hours (Mladinic et al., 2009a). Effects were observed both in the presence and absence of S9 with statistically significant increases in tail intensity at 3.5, 92.8 and 580 µg/ml without S9 and at 580 µg/ml with S9. A modification of the alkaline SCGE assay employing human 8-hydroxyguanine DNA-glycosylase (hOGG1) to detect oxidative damage only indicated statistically significant effects on tail length for treatment with 580 µg/ml with S9. Increases in nuclear abnormalities (nuclear buds and/or nucleoplasmic bridges) were also observed at 580 µg/ml with and without S9 and in micronucleus frequency at 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 (alkaline SCGE 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. A reasonable summation of the results in this publication is that alkaline SCGE effects and other effects such as nuclear abnormalities, early apoptosis, necrosis and oxidative damage were consistently observed at 580 µg/ml.

In addition to mammalian cell studies there are publications reporting positive alkaline SCGE effects for glyphosate in *Tradescantia* flowers and nuclei exposed to up to 700 µM glyphosate (Alvarez-Moya et al., 2011) and in the *E. coli* SOS chromotest for DNA damage conducted on a Roundup BIO GBF (Raipulis J, 2009). Observations of DNA damage in plants exposed to glyphosate are of questionable significance because of the herbicidal nature of glyphosate and the SOS chromotest provides only indirect evidence of DNA damage in a bacterial system.

Overall there appear to be a number of studies in which glyphosate or GBFs have been reported to produce positive responses in DNA damage endpoints of SCE or alkaline SCGE *in vitro* in mammalian cells. Most of these have occurred with exposures to mM concentrations of glyphosate. Although this dose level range is lower than the limit dose of 10 mM recommended for several *in vitro* mammalian cell culture assays (OECD473, 1997; OECD476, 1997; OECD487, 2010), an even lower limit dose of 1 mM was recently recommended for human pharmaceuticals, particularly because of concerns about relevance of positive *in vitro* findings observed at higher dose levels (ICHS2(R1), 2008; Parry et al., 2010). In addition, many of the studies have limitations such as not indicating control of medium pH and not coding 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 alkaline SCGE effects and cytotoxic or apoptotic mechanisms. One recommendation for the *in vitro* alkaline SCGE 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 alkaline SCGE effects (Storer et al. 1996). Other recommendations include conducting experiments to measure DNA double strand breaks to determine if apoptotic process might be responsible for alkaline SCGE effects. Measurement of apoptotic and necrotic incidence were only performed in one publication (Mladinic et al., 2009a) and these measurements indicated both apoptotic and necrotic processes occurring in parallel with observations of alkaline SCGE effects. These direct observations as well as the reported dose responses, consistently suggest that biological effects and cytotoxicity accompany the observations of DNA damage *in vitro* in

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

6.2 In vivo DNA Damage Studies

In the earlier review positive results for DNA strand breakage were reported for mice treated by the i.p. route with glyphosate and GBFs and for the alkaline SCGE endpoint in tadpoles of the frog *Rana catesbiana* exposed to a GBF (Williams et al., 2000).

Only one *in vivo* mammalian DNA damage study was since reported. This report indicated an increase in SCE frequency in bone marrow cells of mice treated with uncharacterized herbazed GBF (Amer et al., 2006). Statistically significant positive effects were only observed at the highest dose level tested (200 mg/kg administered p.o.).

Several recent publications report alkaline SCGE results for GBFs in aquatic species. Three publications reported positive alkaline SCGE results in aquatic vertebrates exposed to Roundup GBFs in water. These publications have a common feature that alkaline SCGE results were reported as visually scored damage category incidence rather than instrumental measurements of properties such as the tail length or tail intensity. In one publication increases in nuclei exhibiting alkaline SCGE visual damage effects were observed in erythrocytes and gill cells of the tropical fish *Prochilodus lineatus* exposed to 10 mg/liter of a Roundup GBF in water (Cavalcante et al., 2008). Results were variable with cell type and incubation; statistically significant positive responses were observed for erythrocytes at 6 hours and 96 hours, but not 24 hours or for branchial cells from the gills at 6 hours and 24 hours. Measurement of erythrocyte micronucleus frequency and nuclear abnormalities did not show statistically significant increases in these endpoints. The concentration used was reported to be 75% of the 96 hour LC50, but trypan blue dye measurements apparently indicated >80% viability of cells used in the alkaline SCGE assays. A second publication reported positive alkaline SCGE results in erythrocytes of the goldfish, *Carasseus auratus*, exposed to 5, 10 and 15 ppm of a Roundup GBF for 2, 4 or 6 days (Cavas and Konen, 2007). Similar effects were observed for other endpoints (micronucleus and nuclear abnormalities). In general, effects increased with concentration and time. This publication did not report toxicity measurements or, more specifically, measurements of cell viability in the population studied. Positive results were also reported in erythrocytes of the European eel, *Anguilla anguilla*, exposed to 58 and 116 µg/liter of a Roundup 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. Measurement of toxicity was not reported for the animals or erythrocytes; however, several endpoints relevant to antioxidant responses and oxidant effects were made in whole blood samples. No statistically significant effects were observed for catalase, glutathione transferase, glutathione peroxidase, glutathione reductase or reduced glutathione content. A large statistically significant increase for thiobarbituric acid reactive substances (TBARS, a measure of lipid peroxidation) was observed for the 115 µg/liter concentration group at 1 day. Statistically significant TBARS increases were not observed at 3 days, but, the 3-day negative control value appeared to be several fold higher than the 1-day value. Negative alkaline SCGE results were reported in cells of freshwater mussel larvae exposed to 5 mg/liter of a Roundup GBF 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 LC50 for this GBF was reported to be 18.3 mg/liter in parallel experiments.

6.3 Significance of DNA Damage Endpoint Results

DNA damage endpoints such as SCE or alkaline SCGE are generally regarded as supplementary to the gene mutation and chromosome effects endpoint categories. DNA damage endpoints 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 alkaline SCGE can be induced by cytotoxicity and cell death processes rather than from DNA-reactive mechanisms.

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 (Benigni, 1989; Bradley et al., 1979; Decuyper-Debergh et al., 1989; Djelic et al., 2006; Eckl et al., 1993; Speit, 1986; Tayama and Nakagawa, 1994; Zeiger et al., 1990). 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 alkaline SCGE effects can be secondary to necrotic or apoptotic processes (Amin et al., 2000; Henderson et al., 1998; Kiffe et al., 2003; Storer et al., 1996; Tice et al., 2000). Several clear specific examples exist of *in vitro* induction of alkaline SCGE 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 alkaline SCGE 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). The reported positive results for vitamin C by Anderson et al. (1994) are interesting because comet effects were observed in the same 1-10 mM concentration range as reported for glyphosate or GBFs in *in vitro* alkaline SCGE assays. Further examples of alkaline SCGE effects of questionable genotoxic biological significance include dietary flavonoids quercitin, 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 also interesting because it suggests responses to surfactants which are typically components of GBFs. As a more specific example, polyoxyethylenealkalylmine (POEA), a surfactant component of some GBFs 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 GBFs inducing DNA damage responses. Such responses would be expected to be associated with cytotoxicity-inducing exposures and exhibit a threshold.
Some data suggest better concordance of the alkaline SCGE 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 alkaline SCGE effects with questionable genotoxicity significance because of negative results for other *in vivo* genotoxic endpoints or carcinogenicity assays or which appear to be due to toxicity. Some examples of positive results for non-carcinogens include thiabendazole, saccharine, tartrazine and ortho-phenylphenol (Brendler-Schwaab et al., 2005). Discordance between carcinogenicity species specificity and *in vitro* alkaline SCGE 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 alkaline SCGE effects produced in lymphocytes of exercising humans that were not accompanied by micronucleus induction (Hartmann et al., 1998)
It is has long been recognized that alkaline SCGE effects, even in *in vivo* studies, can arise from processes that do not involve direct DNA-reactivity such as cytotoxicity and induction of apoptosis (Tice et al., 2000; Hartmann et al., 2003; Burlinson et al., 2007). Concurrent assessment of cytotoxicity is recommended in *in vivo* studies. The reported "gold standard" for cytotoxicity 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 alkaline SCGE effects, they may be used as an additional measure of toxic effects (Smith et al., 2008). With the exception of a mouse bone marrow SCE effect of herbazed GBF all of the reported *in vivo* DNA damage results are in non-mammalian species with limited experience and none of the assays reported evaluations for cytotoxic effects recommended for *in vivo* assays.

7. Human and Environmental Studies

A number of human and environmental studies have been published in or after 2000 where some exposures to GBFs in the studied populations were postulated. These publications are summarized in Table 3.

Table 3. Studies of Human and Environmental Populations with Reported or Assumed Glyphosate Exposure

Exposed Population	Endpoint	Exposures	Result	Reference
Human Studies				
Open field and fruit farmers	Bulky DNA adducts	glyphosate formulation use reported in only 1 of 29 fruit farmers	No effects attributed to glyphosate formulation exposure	Andre V, 2007
Humans in areas where glyphosate formulation is applied	Lymphocyte cytokinesis block micronucleus (CB MN)	Aerial or manual spraying of glyphosate formulation for illicit crop control and sugar cane maturation	Increase in CB MN but no clear relationship to assumed or reported exposures	Bolognesi et al., 2009
Floriculturists	Lymphocyte CB MN	Glyphosate formulation use reported in 21/51 workers with average of 106.5 kg applied	Increase in CB MN but not statistically significant	Bolognesi et al., 2004
Floriculturists	Lymphocyte CB MN	Glyphosate formulation use reported in 57/107 workers. Numerous other pesticides reported as used by a similar number or more of workers	Statistically significant increase in CB MN	Bolognesi et al., 2002
Agricultural workers	Buccal cell micronucleus	Glyphosate formulation use reported along with numerous other pesticides	Statistically significant increase in MN	Bolognesi et al., 2009
Workers exposed to pesticides	Lymphocyte SCE, micronucleus, chromosome aberration (CA)	Glyphosate formulation use reported along with numerous other pesticides	Statistically significant increases in MN and SCE but not chromosome aberrations	Costa et al., 2006
Fruit growers	Lymphocyte Alkaline SCGE; Ames test on urine	Glyphosate use reported in 2/19 1 day before captan spraying and 1/19 on the day of captan spraying	No effects attributable to glyphosate formulation exposure	Lebailly et al., 2003
Agricultural workers	Lymphocyte SCE; buccal cell micronucleus	Glyphosate formulation use reported along with numerous other pesticides	Statistically significant increase in SCE in lymphocytes and micronucleus frequency in buccal cells	Martinez-Valenzuela et al., 2009
Agricultural workers	Lymphocyte CB MN; buccal cell micronucleus	Glyphosate formulation use reported in 16% of one of four populations studied (Hungary)	No statistically significant increases in CB MN or buccal cell micronucleus frequencies	Pastor et al., 2003
Individuals on or near glyphosate spraying	Lymphocyte alkaline SCGE	Glyphosate formulation aerially sprayed within 3 km	Statistically significant increases in damaged cells	Paz-y-Mino C, 2007

Exposed Population	Endpoint	Exposures	Result	Reference
Greenhouse Farmers	Lymphocyte SCE	Glyphosate formulation use reported in 99/102 workers; numerous other pesticides used	Statistically significant increases in SCE	Shaham et al., 2001
Workers exposed to pesticides	Lymphocyte alkaline SCGE	Glyphosate formulation use reported along with numerous other pesticides	Statistically significant increases in damaged cells	Simoniello et al., 2008
Farmers	Lymphocyte CB MN	Glyphosate formulation use reported in 3/11 farmers	Statistically significant increase in micronucleus frequency but not in frequency of binucleated cells with micronuclei	Vlastos D, 2006
Environmental Studies				
Meadow voles living on golf cousres	Blood cell alkaline SCGE; erythrocyte micronucleus	Glyphosate formulation use reported along with numerous other pesticides	Some effects judged possibly related to Daconil® fungicide	Knopper et al., 2005
Fish from dams (various species)	Erythrocyte micronucleus	Glyphosate formulation use reported in adjacent lands along with other pesticides	Higher MN frequencies than normal or expected but no negative concurrent controls used	Salvagni J, 2011

Many of the human studies either found no effects attributable to GBFs or the reported GBF usage by the studied population was too low to be associated with observed population effects (Andre V, 2007; Bolognesi et al., 2004; Lebailly et al., 2003; Pastor et al., 2003; Vlastos D, 2006).

Several other studies did not indicate the extent of usage of specific pesticides but simply listed a large number of pesticides used by the populations (Bortoli et al., 2009; Costa et al., 2006; Martinez-Valenzuela et al., 2009; Simoniello et al., 2008). In other studies, incidence of GBF use by the population studied was significant but high incidence of use of other pesticides was also reported (Bolognesi et al., 2002; Shaham et al., 2001). Even though positive effects were observed in these populations, ascribing these effects to any particular environmental exposure is not scientifically justifiable and such results certainly cannot be considered as definitive evidence for GBF-induced human genotoxic effects.

Two published studies focused on populations believed to be exposed to GBFs by their presence at or near aerial or manual spraying operations. One publication reported induction of alkaline SCGE effects in blood lymphocytes of populations living within 3 km of areas sprayed with glyphosate formulation for illicit crop eradication (Paz-y-Mino C, 2007). The populations studied were relatively small (24 exposed individuals and 21 non-exposed individuals). The sprayed material was reported to be Roundup Ultra, a GBF containing 43.9% glyphosate, polyethoxylated tallowamine surfactant and a proprietary component, Cosmoflux 411F. Specific methods for collection, storage, and transport of blood samples are not described for either the exposed population or control group. The publication also does not indicate that slides were coded for scoring which consisted of visual classification into damage categories and measurement of DNA migration (tail length). There were fairly large differences in ages and sex distribution of the exposed and control populations but these did not appear to be statistically significant. The study reported increases in damaged cell categories and statistically significant increases in DNA migration (tail length) in the presumably exposed population. Interpretation of the results of this study should consider numerous reported signs of toxicity in the exposed population and the reported application rate of 24.3 liters/ha which was stated to be 20 times the maximum recommended application rate. Some of the reported human health effects described by Paz-y-Mino (2007) appear to be consistent with severe

exposures noted in clinical reports of acute poisoning incidents with GBFs and other pesticide formulations (often self-administered) rather than typical bystander exposures (Menkes et al., 1991). Given the considerably favorable general toxicology profile of glyphosate as reported by the WHO/FAO Joint Meeting on Pesticide Residues (WHO/FAO, 2004) and in Williams et al. (2000), factors related to either high surfactant exposure, unusual GBF components in this formulation or other undocumented variables appear to be confounding factors in this study. It appears that the reported alkaline SCGE effects could well have been secondary to the ailments reported in this study population.

A second publication reported results for a blood lymphocyte cytokinesis-block micronucleus study of individuals in areas treated with glyphosate formulation by aerial spraying or manual application (Bolognesi et al., 2009). Although the title of the publication contains the term "agricultural workers", most of the populations studied do not appear to be agricultural workers who are involved in application of GBFs. The human lymphocyte culture and scoring methodology employed in the Bolognesi et al. (2009) study appear to be generally consistent with commonly used and recommended practices for this assay. However, there is a significant question as to how long the blood samples used in the study were stored prior to initiating cultures and this may have affected the micronucleus numbers observed in the different sets of samples and populations. Also, the populations in the aerially sprayed regions had a second sampling a few days after the first sampling and this second sampling was not performed in the control populations. The publication reported a small increase in the frequency of binucleated cells with micronuclei and micronuclei per cell in samples collected from people living in three regions after spraying of GBFs compared with control values of samples collected just before spraying. However, the pattern of the increases did not correlate either with the application rate or with self-reported exposure. The largest post-spraying increase in binuclated cell micronucleus frequency was reported for a population with a much lower glyphosate active ingredient application rate and only 1 of 25 people in this region reported contact with sprayed glyphosate formulation. Increases in binucleated cell micronucleus frequency did not have a statistically significant relationship with self-reported exposure for two other populations. Some interpretative statements in Bolognesi et al. (2009) suggest a small transient genotoxic effect of glyphosate formulation spraying on frequencies of binucleated cells with micronuclei, but other statements indicate that causality of the observed effects could not be determined using reasonable criteria and that lack of exposure data precluded conclusions. This study has a combination of uncontrolled or inadequately characterized variables, such as uncharacterized exposure to "genotoxic pesticides", that would appear to preclude using the data to support any conclusion that exposure to GBFs affects binucleated micronucleus frequencies. Actually, the available data, while certainly limited in nature, support a conclusion that the observed effects do not appear to be attritubable to glyphosate formulation exposure. This conclusion is reinforced by Acquavella et al. (2004), where biomonitoring of agricultural workers applying GBFs reports systemic exposures orders of magnitude below *in vivo* model chromosome aberration and micronucleus study doses, the majority of which were negative for glyphosate and GBFs.

There are two publications related to environmental monitoring for genotoxic endpoints. One study using blood cell alkaline SCGE and micronucleus endpoints was conducted on samples from meadow voles living on or near golf courses where pesticides had been applied (Knopper et al., 2005). Results were significantly inconsistent between two seasons. Although some suggestions of effects were reported, glyphosate was only one of a number of applied pesticides and the effects observed were considered as possibly attributable to exposure to Daconil® fungicide. A second publication reported results for the erythrocyte micronucleus assay applied to fish collected from several dams in Brazil (Salvagni J, 2011). Glyphosate formulation was one of a number of pesticides reported to be used in the area of the dams. No efforts appear to have been made to measure glyphosate or other pesticide concentrations in any of the ten dams from which fish were sampled. This study reported what were considered to be high levels of micronucleated cell frequency but there were no concurrent negative controls. In the absence of these controls the results cannot be interpretted as indicating any effect of pesticide exposure.

Although there have been a fairly large number of human genotoxicity studies reported where there was some exposure to GBFs, the large majority of these studies do not allow any conclusions about possible effects of glyphosate or GBFs because the exposure incidence was low or because there were reported exposures to a large number of pesticides. One report found an increase in alkaline SCGE effects in humans living in or near areas where a GBF was sprayed but that study had a number of methodology reporting and conduct deficiencies and the reported effects could well have been due to toxicity reported in the study population. A second study found some increases in cytokinesis-block micronucleus

frequency in humans possibly exposed to GBFs but the effects were not concordant with application rates or self-reported exposures and thus do not constitute reliable indications of effects for this endpoint in humans exposed to GBFs. Neither of the two environmental monitoring studies in meadow voles or fish provide any reliable evidence of exposures to glyphosate or GBFs or adverse effects resulting from potential exposures to glyphosate or GBFs.

8. *DNA-Reactivity and Carcinogenesis*

As noted in the earlier review, ^{32}P-postlabelling DNA adduct studies in mice did not indicate formation of adducts from glyphosate and questionable evidence of adducts from Roundup GBF administered as a high 600 mg/kg i.p. dose in an unusual dimethylsulfoxide/olive oil vehicle (Peluso et al., 1998; Williams et al., 2000). Another earlier reviewed study reported DNA strand breakage in liver and kidneys of mice injected i.p with glyphosate and Roundup GBF. This study also reported an increase in 8-hydroxydeoxyguanosine (8-OHdG) residues in liver DNA from mice injected with glyphosate but not GBF. Increased 8-OHdG was found in kidney DNA from mice injected with GBF but not glyphosate (Bolognesi et al., 1997; Williams et al., 2000). No new direct studies of DNA reactivity of glyphosate or GBFs were encountered in publications since 2000. One publication did report on studies in mice to further investigate toxic effects and 8-OHdG levels associated with the routes, vehicles and dose levels employed in earlier ^{32}P-postlabelling and DNA strand breakage and 8-OHdG studies (Heydens et al., 2008). This publication reported that high i.p. dose levels of GBF induced significant liver and kidney toxicity that were not observed with oral administration. Statistically significant increases in 8-OHdG were not observed in this study under the same conditions as employed by the earlier study. The dimethylsulfoxide/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 and which were produced by the i.p. route of exposure to very high dose levels. The enhancement of toxicity by the unusual dimethylsulfoxide/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.

Carcinogenicity is not a direct endpoint for genotoxicity but it is one of the possible consequences of genotoxicity and, conversely, lack of carcinogenicity in well-conducted experimental studies provides some evidence that a significant genotoxic mode of action is not operating *in vivo*. The earlier review of glyphosate concluded that it was not carcinogenic in mouse or rat chronic studies and notes that glyphosate was not considered carcinogenic by numerous regulatory agencies and scientific organizations (Williams et al., 2000).

9. *AMPA and POEA*

In addition to glyphosate and GBFs, the earlier review included information on the toxicity and genotoxicity of the major environmental breakdown product of glyphosate, aminomethylphosphonic acid (AMPA), and what was at that time a common GBF surfactant mixture of polyethoxylated long chain alkylamines synthesized from animal-derived fatty acids (polyethoxylated tallow amine, tallowamine ethoxylate, POEA). Today a wide variety of surfactant systems are employed by different companies for different regions and end uses.

In the earlier review, summarized genotoxicity results for AMPA included negative results in the Ames/*Salmonella* bacterial reversion assay, an *in vitro* unscheduled DNA synthesis assay in primary hepatocytes and a mouse bone marrow erythrocyte micronucleus assay (Williams et al., 2000). One publication of AMPA genotoxicity results was observed subsequent to 2000. In this publication analytical grade AMPA was reported to have positive effects in several assays including an alkaline SCGE endpoint in cultured mammalian Hep-2 cells, a chromosome aberration endpoint in cultured human lymphocytes and in a mouse bone marrow erythrocyte micronucleus assay (Manas et al., 2009a). Experimental limitations in the conduct of the alkaline SCGE assay included no inclusion of mammalian metabolic activation and no reported control of medium pH even though relatively high concentrations of AMPA acid (2.5-10 mM for 4 hours) were used. Although nucleoid images were analyzed with software rather

than visual analysis the methodology doesn't indicate that slides were coded and there may have been a visual judgment component in selection of images for analysis. The positive results were statistically significant increases in tail length, % DNA in tail and tail moment at 4.5 to 7.5 mM AMPA. The human lymphocyte chromosome aberration assay also did not employ an exogenous mammalian metabolic activation system but control of medium pH and blind scoring of slides were reported for this assay. A small increase in chromosome aberrations per 100 metaphases was observed in cells exposed to 1.8 but not 0.9 mM AMPA for 48 hours. The increase was marginally significant ($p<0.05$) and no statistically significant increases were observed for any specific chromosome aberration category. Although number of cells with aberrations are commonly used to describe results from *in vitro* chromosome aberration assays (OECD473, 1997) these data were not presented. Given the marginal significance, these omissions are a significant limitation in interpreting the results. Positive results were also reported for a mouse micronucleus bone marrow assay in mice administered 2 x 100 mg/kg or 2 x 200 mg/kg i.p at 24 hour intervals. The methodology description did not indicate that slides were coded for analysis in this assay. Results were reported as a statistically significant increase from a negative control value of 3.8/1000 micronucleated erythrocytes to 10.0 and 10.4/1000 micronucleated erythrocytes in the 2 x 100 and 2 x 200 mg/kg dose groups, respectively. These data do not indicate a reasonable dose response and a third dose level was not employed as recommended for this assay (OECD474, 1997). The publication indicates micronucleus scoring results for "erythrocytes" and not polychromatic or immature erythrocytes as would be appropriate for the acute dose protocol employed. Although this might be an inadvertent error in methodology description the term polychromatic erythrocytes was used in the methods section and PCE was used in the results table to describe scoring of PCE/NCE ratio.

The reported positive effects for AMPA in the *in vitro* studies are not concordant with *in vitro* results for other endpoints or the lack of genotoxic structural alerts in the structurally similar parent molecule moieties from DEREK *in silico* analysis. The alkaline SCGE effect could be due to cytotoxicity, especially considering the relatively high dose levels employed (close to the 10 mM upper limit dose) and the lack of indication of pH control. Although limited cytotoxicity (>80% viability) was reported using the trypan blue exclusion method this endpoint may grossly underestimate cytotoxic effects observed with other endpoints (Fellows and O'Donovan, 2007).

The *in vitro* chromosome aberration assay positive result was of low magnitude and was of particularly questionable significance, considering the lack of statistical significance for any individual chromosome aberration category and that the results for number or percent of cells with chromosome aberrations were not reported.

There is a clear discordance in results for AMPA in the mouse bone marrow micronucleus assay. In the earlier review negative results were reported for AMPA in a mouse bone marrow micronucleus assay conducted with dose levels up to 1000 mg/kg i.p. (Williams et al., 2000) The maximum dose level was much higher than those used by Manas et al. (2009a) Although Manas et al. used a protocol with two doses separated by 24 hours and a single harvest time, this protocol would not be expected to give different results than a single dose with multiple harvest times, particularly when the maximum single dose was much higher (OECD474, 1997). PCE/NCE ratio data from the Manas et al. (2009a) study do not indicate that there were detectable bone marrow toxic effects observed under the conditions of their study. It appears possible that Manas et al. may have inappropriately scored erythrocytes for micronuclei instead of polychromatic erythrocytes, but if this is the case lower sensitivity rather than higher sensitivity would be expected. These limitations suggest the possibility that the aberrant result might be that of Manas et al. (2009a) but further studies might be necessary to resolve the discordance.

The earlier review reported negative results for POEA in an Ames/*Salmonella* assay (Williams et al., 2000). No other genotoxicity results were reported for POEA individually but numerous genotoxicity results were presented, as described earlier, for GBFs containing POEA. Examination of subsequent literature for this review did not produce any new publications reporting genotoxicity results for POEA as an individual test material (i.e. not as a glyphosate formulation). However, there were some publications confirming that POEA can be a significant contributor to toxicity of GBFs and that it exhibits biological effects consistent with surfactant properties. These POEA effects have been noted in aquatic species of several taxa (Folmar et al., 1979; Moore et al., 2011; Perkins et al., 2000; Tsui and Chu, 2003; Wan et al., 1989). As noted earlier, experiments with a POEA-containing formulation without glyphosate administered i.p. in DMSO/olive oil vehicle to mice produced the same severe liver and kidney toxicity as a GBF indicating that the toxicity primarily resulted from the formulation components rather than

glyphosate (Heydens et al., 2008). Similarly, dose-response curves were superimposed for an *in vitro* system evaluating a GBF and the same formulation without glyphosate present (Levine et al., 2007). A transcription profiling study of a Roundup GBF in yeast produced responses similar to those produced by detergent and oil treatments, and glyphosate alone did not produce effects at equivalent concentrations (Sirisattha et al., 2004). Effects on mammalian cells consistent with membrane disruption and consequent cytotoxicity were observed for POEA (Benachour and Seralini, 2009).

10. Genotoxicity Weight of Evidence

The earlier review 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 this analysis was that glyphosate and Roundup GBFs 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, a large number of genotoxicity studies have been conducted with glyphosate and GBFs. For gene mutation, one of the two primary endpoint categories with direct relevance to heritable mutation, one subsequent publication contains a summary of results from a bacterial gene mutation endpoint assay (Ames/*Salmonella* bacterial reversion assay). Although there were very significant limitations to the information published, the negative result is consistent with the majority of negative results reported for glyphosate and GBFs in Ames/*Salmonella* bacterial reversion assays. Another publication reported results for a *Drosophila* wing spot assay of glyphosate. Results were negative or inconclusive in this assay for crosses that would have detected gene mutation as loss of heterozygosity. The new results provide some support to reinforce the earlier conclusion that glyphosate and GBFs are not active for the gene mutation endpoint category.

The second primary endpoint category with direct relevance to heritable mutation is chromosome effects. The earlier review noted mixed results for two *in vitro* chromosome effects assays in mammalian cells but concluded that the most reliable result was the negative assay. A number of *in vitro* mammalian cell chromosome aberration or micronucleus assay results have been subsequently published using bovine or human lymphocytes. These assays suffer from some technical limitations in conduct or reporting of methodology that frequently included failure to indicate control of medium for pH and failure to indicate coding of slides for visual scoring. Both positive and negative results are reported in these assays. A large preponderance of results in the absence of an exogenous mammalian metabolic activation system were negative up to high (mM) dose levels that were toxic or close to toxic levels observed in parallel experiments. The exceptions were a weak and inconsistent response reported in two publications from the same laboratory and a positive response for the uncharacterized formulation, herbazed. In addition to these findings in mammalian cells negative results were also reported for Roundup GBF in an onion root tip assay conducted without exogenous mammalian metabolic activation. Thus, the preponderance of evidence from assays not employing an exogenous mammalian metabolic activation system indicates that glyphosate and GBFs are not structural chromosome breakage inducers (clastogenic) in *in vitro* mammalian chromosome aberration or micronucleus assays.

Two publications from one laboratory reported an increase in micronucleus frequencies for glyphosate in *in vitro* cultured mammalian cells in the presence of an exogenous S9 metabolic activation system (Mladinic et al., 2009a; Mladinic et al., 2009b). An enrichment for centomeric-containing micronuclei suggested that the increased micronuclei observed in these studies were derived from aneugenic processes, probably mediated through toxicity, rather than chromosome breakage. Thus, these two reports of weak micronucleus responses in the presence of exogenous mammalian metabolic activation appear to result from toxicity-associated aneugenic rather than clastogenic mechanisms. A number of other gene mutation and *in vitro* chromosome effect genotoxicity studies are negative with exogenous metabolic activation which supports the conclusion that the weight of evidence does not indicate a DNA-reactive clastogenic activity in *in vitro* assays using mammalian cells.

All except one of a number of *in vivo* mouse bone marrow chromosome aberration or micronucleus assays of glyphosate and GBFs were reported as negative in the earlier review. In the updated review both

positive and negative results were reported for glyphosate and GBFs in these types of assays. Many of these studies had limitations or deficiencies compared to international guidelines with the most common and significant being no indication of slide coding for visual scoring. Four publications from three laboratories reported negative results in mouse bone marrow erythrocyte micronucleus assays of glyphosate and GBFs which are consistent with the earlier reviewed studies. These studies used high, peri-lethal dose levels administered by the i.p. or oral routes.

Two publications from two laboratories reported positive results for glyphosate and GBFs in the mouse bone marrow erythrocyte micronucleus assay. One positive result for glyphosate was encountered using dose levels and routes that were similar to those employed in the negative glyphosate studies in the same assay system. The publication reporting this result indicates that erythrocytes rather than polychromatic erythrocytes were scored which would be inappropriate for the treatment protocol but it is possible that this is a misreporting of what types of cells were actually scored. Although there is no definitive explanation for the discordance, the preponderance of mouse bone marrow erythrocyte micronucleus studies of glyphosate are clearly negative. The reported positive result for Roundup GBF is discordant with a number of negative results for Roundup or other GBFs conducted at higher dose levels. The most unique feature of this study was the use of dimethylsulfoxide as a vehicle. The preponderance of mouse bone marrow erythrocyte micronucleus studies for Roundup and other GBF studies is negative.

Positive results were reported in an unusual test system (rabbit) and route (drinking water), but water intake was not reported and effects may therefore be attributable to dehydration. Furthermore, most of the effects were on endpoints not usually considered as indicators of clastogenicity and structural chromosome aberration. One laboratory reported positive results for chromosome aberration effects in bone marrow and spermatocytes after extended dosing. However, the herbazed formulation test material was not characterized.

While more discordant results in the important *in vivo* mammalian chromosome effect assay category have been reported in publications subsequent to the earlier 2000 review the preponderance of evidence continues to indicate that glyphosate and GBFs are not active in this category of endpoint.

Several *in vivo* erythrocyte micronucleus assay results for GBFs in non-mammalian systems (fish and caiman eggs) have been published since the earlier review. These test systems have relatively little experience and are largely unvalidated in comparison to the mouse bone marrow erythrocyte micronucleus assay. Two publications report negative results and two publications report positive results in different fish species and there is no definitive explanation for the discordance. Both the positive and negative studies employed maximum dose levels that were toxic or close to toxic dose levels. One possible explanation for the discordance is that the positive effects were associated with toxicity that only occurred beyond an exposure threshold and over a fairly narrow dose range. Positive results in hatchlings derived from caiman eggs exposed to Roundup formulation are given relatively little weight because of extremely limited experience with this assay system and because of significant questions about how DNA damage effects induced in embryos can persist and be evident in cells of hatchlings after several weeks and numerous cell divisions. The reported weak and inconsistent response in one of four crosses for somatic recombination in the *Drosophila* wing spot assay is also accorded relatively low weight. These non-mammalian test systems are generally considered of lower weight for predicting mammalian effects than mammalian test systems. Also, the environmental significance of effects for GBFs should consider the relationship between concentrations or exposures producing effects and likely environmental concentrations or exposures. This is particularly important if the effects are produced by threshold mediated toxic processes.

There have been a significant number of publications since the earlier review of results for assays in the DNA damage category with some SCE and a large number of alkaline SCGE endpoint publications. In general, the DNA damage endpoint category is considered supplementary to the gene mutation and chromosome effect categories because this endpoint category does not directly measure heritable events or effects closely associated with heritable events. Regulatory genotoxicity testing recommendations and requirements focus on gene mutation and chromosome effect endpoints for initial core testing, particularly for *in vitro* testing (Cimino, 2006; Eastmond et al., 2009; ICHS2(R1), 2008). This consideration is underscored by the observation of some cases of compounds where positive effects are observed in these assays that are not observed for gene mutation or chromosome effect assays. Also, there are numerous examples of responses in these endpoints that do not appear to result from mechanisms of direct or

metabolite DNA-reactivity. The unique response consideration is reinforced in this data set by observations of responses in DNA damage endpoints but not in chromosome effect endpoints.

Many DNA damage endpoint assays of glyphosate or GBFs have produced positive results at high, toxic or peri-toxic dose levels for the SCE and alkaline SCGE endpoints in a variety of test systems including cultured mammalian cells, several aquatic species and caiman eggs. The only new report of positive *in vivo* mammalian DNA damage effects are for an uncharacterized formulation, herbazed. There are several examples of negative results for a chromosome aberration or micronucleus endpoint and positive results for the alkaline SCGE or SCE endpoint in the same publication (Cavalcante et al., 2008; Manas et al., 2009b; Mladinic et al., 2009a; Sivikova and Dianovsky, 2006). These examples confirm the impression that the DNA damage endpoints are not necessarily predictive of heritable mutation effects and are also consistent with the DNA damage endpoints reflecting toxic effect mechanisms. While the number of reported positive responses in these endpoints does suggest that effects in these endpoints can be induced by glyphosate or GBFs, comparison with results for gene mutation and chromosome effects endpoints, examination of the dose response and association of the effects with toxic endpoints indicates that these effects are likely secondary to toxicity and are threshold mediated. Surfactants in GBFs increase toxicity compared to the active ingredient of glyphosate salts and are shown to induce effects such as membrane damage and oxidant stress which are likely capable of inducing DNA damage effects at cytotoxic doses. These factors as well as other considerations presented in Section 6.3 indicate that these DNA damage effects have negligible significance to prediction of hazard or risk at lower and more relevant exposure levels.

Most of the human studies do not provide interpretable or relevant information regarding whether there are *in vivo* human genotoxic effects of GBFs because the reported incidence of glyphosate formulation exposure in the population was low or because there were reported exposures to a relatively large number of pesticides. Two studies with focus on glyphosate exposure through presence in or near areas of glyphosate formulation spraying found increases in the DNA damage alkaline SCGE endpoint. In one study clinical signs of toxicity were reported in the population and spraying concentrations were reported to be many times the recommended application rate. Given the nature of the endpoint a reasonable interpretation is that effects might well be due to the overt toxicity that was reported in the publication. This would be a threshold mediated, non-DNA reactive mechanism and is consistent with experimental system results showing alkaline SCGE effects in animals exposed to high levels of formulation components. The low weight of evidence for significant genotoxic hazard indicated by this particular endpoint in human monitoring is reinforced by findings that exercise induces alkaline SCGE effects in humans (Hartmann et al., 1998). The other study found increases in binucleated micronucleated cell frequency in population in spraying areas but the increases were not consistent with spraying levels or self-reported exposure. These latter observations are not consistent with the study presenting clear evidence of GBF effects on this endpoint. In sum, the available human data do not provide any clear indications that exposed humans are substantially different in response than mammalian animal models or that exposure to GBFs produces DNA-reactive genotoxicity.

Carcinogenicity is an adverse effect that is a possible consequence of genotoxic and mutagenic activity. Conversely, lack of carcinogenicity in properly conducted animal models is supportive for lack of significant *in vitro* mammalian genotoxicity. The updated review provides one new study of glyphosate formulation which is negative for either initiator or complete carcinogenesis activity which provides additional evidence to reinforce the conclusion from earlier mammalian carcinogenicity assays that glyphosate and GBFs are non-carcinogenic. These findings support the conclusion that glyphosate and GBFs do not have *in vivo* mammalian genotoxicity or mutagenicity.

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 and environmental exposure levels. A study of farmers indicated a maximum estimated systemic glyphosate dose of 0.004 mg/kg for application without protective equipment and a geometric mean of 0.0001 mg/kg (Acquavella et al., 2004). When compared with *in vivo* mammalian test systems that utilize glyphosate exposures on the order of 50-300 mg/kg, the margins of exposure between the test systems and farmers is 12,500-75,000 for the maximum farmer systemic exposure and 0.5-3 million for the geometric mean farmer systemic exposure. These margins are quite substantial, especially considering that many of the *in vivo* genotoxicity studies are negative. Assuming reasonable proportionality between exposure to glyphosate and GBF ingredients, similar large margins of exposure would exist for GBF components. The

margins of exposure compared to *in vitro* mammalian cell exposures are estimated to be even larger. Assuming uniform distribution, the systemic concentration of glyphosate from the Aquavella et al. (2004) farmer biomonitoring study would be on the order of 24nM for the maximum and 0.59 nM for the geometric mean exposure. A typical maximum *in vitro* mammalian exposure of 1-5 mM represents a margin of exposure of 42,000-211,000 for the maximum farmer exposure and 1.7-8.4 million for the geometric mean farmer systemic exposures, respectively.

Overall, the weight of evidence of the studies considered in the earlier review as well as the studies considered in this review indicates that glyphosate and GBFs are not genotoxic in the two general endpoint categories most directly relevant to heritable mutagenesis, gene mutation and chromosome effects. This conclusion results from a preponderance of evidence; however, there are reports of positive discordant results in both endpoint categories. The new studies considered in this review provide some evidence for DNA damage effects induced by high, toxic exposures, particularly for the alkaline SCGE endpoint and for GBFs containing surfactant. Several considerations, including the lack of response in other endpoint categories, suggest that these effects result from toxic and not DNA-reactive mechanisms and that they do not indicate *in vivo* genotoxic potential under normal exposure levels.

Regulatory and authoritative reviews of glyphosate supporting registrations and registrations in all regions of the world over the last 40 years have consistently determined that glyphosate is nongenotoxic (Commission, 2002; EPA, 1993; WHO/FAO, 2004) (AVPMA, 2010). Scientific publications contrary to these regulatory reviews should be evaluated using a weight of evidence approach with consideration for reliability of the assay used and data quality presented.

Author(s)	Year	Study title
Alvarez-Moya, C., Silva, M.R., Arambula, A.R.V., Sandoval, A.I., Vasquez, H.C., Gonzales Montes, R.M.	2011	Evaluation of genetic damage induced by glyphosate isopropylamine salt using *Tradescantia* bioassays Genetics and Molecular Biology Volume: 34 Number: 1 Pages: 127-130

Abstract*

Glyphosate is noted for being non-toxic in fishes, birds and mammals (including humans). Nevertheless, the degree of genotoxicity is seriously controversial. In this work, various concentrations of a glyphosate isopropylamine salt were tested using two methods of genotoxicity assaying, *viz*., the pink mutation assay with *Tradescantia* (4430) and the comet assay with nuclei from staminal cells of the same plant. Staminal nuclei were studied in two different forms, namely nuclei from exposed plants, and nuclei exposed directly. Using the pink mutation assay, isopropylamine induced a total or partial loss of color in staminal cells, a fundamental criterion utilized in this test. Consequently, its use is not recommended when studying genotoxicity with agents that produce pallid staminal cells. The comet assay system detected statistically significant ($p < 0.01$) genotoxic activity by isopropylamine, when compared to the negative control in both the nuclei of treated plants and directly treated nuclei, but only the treated nuclei showed a dose-dependent increase. Average migration in the nuclei of treated plants increased, when compared to that in treated nuclei. This was probably due, either to the permanence of isopropylamine in inflorescences, or to the presence of secondary metabolites. In conclusion, isopropylamine possesses strong genotoxic activity, but its detection can vary depending on the test systems used.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate (N-(phosphonomethyl)-glycine)
Active substance(s):	Glyphosate
Source of test items:	Aldrich
Lot/Batch #:	09816PE
Purity:	Glyphosate: 96%

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / organism:

Species:	*Tradescentia*
Strain:	Clone (4430) (hybrid *T. subacaulis X T. hirsutiflora*)
Source:	Not reported
Growth conditions:	Daytime temperature: 22°C Night-time temperature: 16-18°C

4. Test methods:

Pink mutation assay:	Assessment of DNA-damage in nuclei from treated plants
Comet assay:	Assessment of DNA-damage in nuclei from treated plants (in vivo assay), or in nuclei from untreated plants exposed in vitro

(in vitro assay)

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Treatment: Plants:
30 inflorescences, corresponding to about 15 flowers (1500 to 3000 stamen hairs), were immersed for 3 h in 250 mL of test substance solution, or negative or positive controls. The test was carried out in duplicate. After exposure inflorescences were washed with distilled water and placed in Hoagland´s solution until further processing.

A part of the inflorescences were used for the pink mutation assay, the other part were used for the comet assay.

Nuclei from untreated plants:
After slide preparation (as described below) slides with nuclei extracted from untreated plants were exposed for 3 h at 25°C to the test substance preparations or controls, washed and stored at 4°C.

Dose levels: 0.7, 0.07, 0.007, 0.0007 mM; vehicle used for preparation not reported

Negative control: Hoaglands solution

Positive control: 1 mM nitrosodiethylamine (NDEA) or 1 mM ethylmethane sulfonate. (Reporting deficiency: in the method section of the report NDA is positive control, in the figure in the results section EMS is positive control!)

Test conditions: Pink mutation assay:

Based on the results of the assay, the authors qualified the assay as unsuitable for Genotoxicity assessment of glyphosate. Therefore this assay in not further described.

Comet assay:

1) Extraction of staminal hair cell nuclei from treated or untreated plants

The stamens of ten treated flowers for each experimental point, obtained on the 6th day after treatment, were homogenised for 2 min using a mortar and Honda buffer (0.44 M sucrose, 2.5% Ficoll (type 400), 5% Dextran-T-40, 25 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 2.5 % Triton-X-100). The homogenate was filtered and the nuclei separated by centrifugation. Nuclei were washed 3 x in washing solution (sucrose 0.4 M, Tris-Base 50 mM, $MgCl_2$, pH 8.5) and resuspended in 200 µL of the same solution. Slides for electrophoresis were prepared according to Singh *et al*., 1988 [Exp. Cell Res., 175, 184-191].

Nuclei from untreated plants were prepared accordingly, using stamens from 10 untreated flowers.

2) Electrophoresis

Slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1 % sodium lauryl sarcosine, 1 % Triton X-100, and 10 % DMSO, pH 10) for 1 h at 4°C, then placed in a horizontal electrophoresis system with a high pH buffer (30 mM NaOH, 1 mM Na_2EDTA, pH 13) for 45 min prior to electrophoresis. Electrophoresis were carried out for 15 min at 1.0 V/cm and approx. 200 mA.

3) Microscopy

Slides were washed, neutralised and stained with ethidiumbromide. After further washing coverslips were added and the slides were evaluated using a fluorescence microscope. Nuclei were observed at 40 x magnification, and migration was determined by visual scoring of tail length, according to published protocols.

Replicates per dose level: 2

5. Observations/analyses:

Measurements: Comet assay: tail length, DNA migration

Statistics: The data obtained were submitted to one-way analysis of variance testing (ANOVA). Dunnett´s test was used for comparing the negative control with data from the nuclei of exposed plants and the exposed healthy ones.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Exposure conditions of plants (immersion) not representative for glyphosate. Inappropriate test model as herbicides are toxic to plants. Presentation of results not sufficient for assessment. Reporting deficiencies (e.g. positive controls)

2. Relevance of study: **Not relevant** (Due to reliability, and exposure conditions of plants and inappropriate test model.).

3. Klimisch code: **3**

Author(s)	Year	Study title
Bolognesi, C. Bonatti, S. Degan, P. Gallerani, E. Peluso, M. Rabboni, R. Roggieri, P. Abbondandolo, A.	1997	Genotoxic activity of glyphosate and its technical formulation roundup Journal of Agricultural and Food Chemistry Volume: 45 Pages: 1957-1962

Abstract*

Glyphosate (N-phosphonomethylglycine) is an effective herbicide acting on the synthesis of aromatic amino acids in plants. The genotoxic potential of this herbicide has been studied: the results available in the open literature reveal a weak activity of the technical formulation. In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. Swiss CD1 mice were treated intraperitoneally with test substances, and the DNA damage was evaluated by alkaline elution technique and 8-hydroxydeoxyguanosine (8-OHdG) quantification in liver and kidney. The chromosomal damage of the two pesticide preparations was also evaluated in vivo in bone marrow of mice as micronuclei frequency and in vitro in human lymphocyte culture as SCE frequency. A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances in vivo and in vitro. A weak increment of the genotoxic activity was evident using the technical formulation.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Active substance(s):	Glyphosate
CAS-No.:	1071-83-6
Source:	Societa Italiana Chimici, Rome, Italy
Purity:	99.9 %
Test item:	Roundup ®
Active substance(s):	Glyphosate
Concentration:	30.4% glyphosate
Source:	Monsanto Italiana, Milan, Italy

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Primary cell culture:	Human lymphocytes
Source:	Heparinised veneous blood samples from two healthy female donors.
Culture conditions:	Whole blood (0.5 mL) was added to 4.5 mL of RPMI 1640 medium supplemented with 17% foetal bovine serum. After addition of 50 µL phytohemoaggluthin (PHA) and 50 µL of 1 mM bromodeoxyuridine (BUdR), cultures were incubated in

	complete darkness at 37 °C.
Animals:	
Species:	Mice
Strain:	Swiss CD1
Source:	Charles River, Como, Italy
Age at dosing:	8-10 weeks
Sex:	Male
Number of animals/group:	3
Weight at dosing:	30 - 40 g
Acclimation period:	Not reported
Diet/Food:	Not reported
Water:	Not reported
Housing:	Not reported
Environmental conditions:	Not reported

4. Test methods:

GLP:	No (for all tests)
***In vitro* sister chromatide exchange (SCE) test:**	Assessment of cytogenicity
Guideline:	None
Guideline deviations:	Not applicable
Dose levels:	Glyphosate: 0, 0,33, 1, 3, 6 mg/mL Roundup: 0, 0.1, 0.33 mg/mL Solvent used for preparation not reported.
Positive control:	None
Negative control:	Culture medium
Test conduct:	24 h after PHA stimulation of the cultured lymphocytes the test substances were added and cultured were further incubated for 48 h. Two hours before determination, 75 µL of Colcemid was added. At termination, 72 h from onset of culture, slides were prepared according to standard methods and stained.
Exposure duration:	Last 48 h of the culture duration of 72 h
Replicates per dose level:	2
Number of cells analysed	At least 50 metaphases were scored for each experimental point by two observers.
***In vivo* alkaline elution assay:**	Assessment of DNA damage
Guideline:	None
Guideline deviations:	Not applicable
Dose levels:	Glyphosate: 300 mg/kg bw Roundup: 900 mg/kg bw (≅ 270 mg/kg bw glyphosate)
Test substance preparations:	Test substances were prepared in physiological saline. The pH of each solution was checked and adjusted to pH 7.0 before treatment.
Positive control:	None

Negative control:	Yes, but details not provided
Conduct of test:	Groups of 3 male mice were treated by i.p. injection with a single dose of the test substance preparations or control. Animals were sacrificed 4 and 24 hours after the injection. Liver and kidney were removed and processed to obtain crude nuclei free from adhering tissues. These nuclei further processed and subjected to alkaline elution assay.
Exposure duration	4 h and 24 h
Replicates per dose level:	3
8-OHdG-levels:	Assessment of oxidative DNA damage
Guideline:	None
Guideline deviations:	Not applicable
Dose levels:	Glyphosate: 300 mg/kg bw
	Roundup: 900 mg/kg bw (≅ 270 mg/kg bw glyphosate)
Test substance preparations:	Test substances were prepared in physiological saline. The pH of each solution was checked and adjusted to pH 7.0 before treatment.
Positive control:	None
Negative control:	Yes, but details not provided
Conduct of test:	Same as described for the alkaline elution assay (see above).
Exposure duration	4 h and 24 h
Replicates per dose level:	3
Tissue sampling and processing:	Livers and kidneys were removed and homogenised in 5 mL PBS. Nuclei were obtained by centrifugation and further processed for DNA extraction. Aliquots of DNA are hydrolised with Nuclease P and alkaline phosphatise, and filtered through cellulose acetate filter units (0.22 mm).
***In vivo* Micronucleus test (MNT):**	Assessment of cytogenicity
Guideline:	None
Guideline deviations:	Not applicable
Dose levels:	Glyphosate: 300 mg/kg bw
	Roundup: 450 mg/kg bw (≅ 135 mg/kg bw glyphosate)
Positive control:	Methyl methanesulfonate (MMS)
Negative control:	Not reported
Animals per dose group:	3
Application:	i.p. injections at 24 h interval
Number of treatments:	2 (test substance groups); 1 (control groups)
Sacrifice:	6, 24 h after the second injection
Sampling and sample processing:	Bone marrow smears were prepared from both femoral bones following the method described by Schmid (1975) with minor modifications.

5. Observations/analyses:

***In vitro* SCE**	
Measurements:	SCEs were determined in at least 50 metaphase cells per culture

***In vivo* alkaline elution assay**

Measurements: DNA elution rate. Fluorometric determination of DNA was performed with Hoechst 33258 reagent.

Results were expressed as elution rate constant K
K(mL^{-1}) = -ln fraction of DNA retained on filter / eluted volume

8-OHdG-levels

Measurements: Approximately 80 µg of DNA per sample is injected in HPLC for 8-OHdG determination.

The separation of 8-OHdG and normal deoxynucleosides is performed in a LC-18-DB column (Supelco, 75 x 4.6 mm) equipped with an LC-18-DB guard column cartridge. UV-detection was accomplished at 254 nm, and electrochemical analysis was carried out by a pulsed electrochemical detector.

The 8-OHdG levels are referred to the amount of deoxyguanidin (dG) detected by UV-absorbance at 254 nm. The amount of DNA is determined by a calibration curve vs known amounts of calf thymus DNA.

8-OHdG-levels are expressed as the number of 8-OHdG-adducts per 10^5 dG bases.

***In vivo* MNT**

Mortality/clinical signs: Not reported

Measurements: In order to score micronuclei about 1000 erythrocytes per animal were analysed.
To evaluate bone marrow toxicity, 1000 erythrocytes were counted and the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated

Statistics for all tests: The standard deviation and the nonparametric test of Mann-Whitney were used for the statistical analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not reliable**

Comment: Basic data given, however, the study is performed with methodological and reporting deficiencies (only data without metabolic activation generated in *in vitro* tests, no positive controls included in *in vitro* SCE and *in vivo* experiments, in some experiments only two test substance concentrations tested)

2. Relevance of study: **Not relevant** (Due methodological and reporting deficiencies data considered to be supplemental information. i.p. exposure route is not relevant for human exposure)

3. Klimisch code: **3**

Author(s)	Year	Study title
Bolognesi, C., Perrone, E., Landini, E.	2002	Micronucleus monitoring of a floriculturist population from western Liguria, Italy Mutagnesis Volume: 17 Number: 5 Pages: 391-397

Abstract*

A biomonitoring study was carried out to investigate whether exposure to complex pesticide mixtures in ornamental crop production represents a potential genotoxic risk. Exposed and control subjects were selected in western Liguria (Italy). The area was chosen for its intensive use of pesticides. The main crops produced were roses, mimosas, carnations and chrysanthemums, as ornamental non-edible plants, and tomato, lettuce and basil, as edible ones. The levels of micronuclei (MN) were analysed in peripheral blood lymphocytes of 107 floriculturists (92 men and 15 women) and 61 control subjects (42 men and 19 women). A statistically significant increase in binucleated cells with micronuclei (BNMN) was detected in floriculturists with respect to the control population (4.41 +/- 2.14 MN/1000 cells versus 3.04 +/- 2.14, $P < 0.001$). The mean number of BNMN varied as a function of sex and age. Smoking habit had no effect on MN frequency. A positive correlation between years of farming and MN frequency in peripheral blood lymphocytes was observed ($r = 0.30$, $P = 0.02$). The conditions of exposure were also associated with an increase in cytogenetic damage, with a 28% higher MN frequency in greenhouse workers compared with subjects working only outdoors in fields. Workers not using protective measures during high exposure activities showed an increase in MN frequency. Our findings suggest a potential genotoxic risk due to pesticide exposure.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	≥ 50 pesticides
Active substance(s):	≥ 50, including glyphosate
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	Exposed group: 17-59 y; non-exposed: 16-53 y
Sex:	Exposed group: 92 male, 15 females non-exposed group: 41 males, 19 females
Smoking habits:	Exposed group: 23 smokers, 36 former smokers, 48 non-smokers Non-exposed group: 20 smokers, 19 former smokers, 22 non-smokers

Persons per group:	107 exposed; 61 non-exposed (control)
Exposure duration:	2-70 years (mean 27.8 ± 15.5)

4. Test system:

Study type:	Epidemilogical study for cytogenicity – Micronucleus assay
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Duration of study:	1 year
Application rate:	Not specified
Persons per group:	107 exposed; 61 non-exposed (control)
Application technique:	Not specified
Mixing/loading performed:	Yes: 88 No: 19
Use of personal protective equipmet (PPE):	Yes: 90 No: 17
Cultivation conditions:	Greenhouses: 19 Open field: 49 Both: 39
Crops:	Ornamentals: 81 Vegetables & ornamentals: 26
Exposure conditions:	75.7 % harvesting ornamentals 24.3% harvesting of ornamentals & vegetables 82.2% preparing pesticides
Blood sampling:	Blood samples were obtained from each subject by venipuncture. All blood samples were collected in sterile sodium heparin tubes. The specimen were received in the laboratory within a few hours of collection and were processed immediately.
Cell cultures:	Whole blood was added to 4.5 mL of RPMI 1640 complete medium with 10 % FCS, and 1% phytohaemoagglutinin. Cells were cultured for 72 hours at 37°C, with cytochalasin B being added after 44 h (concentration: 6 µg/mL). At the end of the incubation period, whole blood cultures were centrifuged, washed, and cells were fixed twice in cold fixative (methanol : acetic acid 3:1) for 20 min at room temperature. Samples for microscopic evaluation were loaded onto wet slides, air dried and stained with 3% Giemsa.

5. Observations/analyses:

Questionnaire:	All subjects. The following information was provided: Demographic information, personal data, smoking habits, history of recent illness and medical treatment. Exposed group: in addition, kind of crops handled, pesticide use, exposure duration, work activity, protective measures
Microscopic micronuclei determination:	2000 binucleated lymphocytes with preserved cytoplasm were scored for each subject on coded slides. The number of binucleated cells with micronuclei (BNMN)

were determined.

Statistics. Parametric and non-parametric statistical test were used. Student´s t-test for independent samples was applied to detect differences in the mean of BNMN in the exposed and non-exposed subjects. Differences among the group means were evaluated by non-parametric Mann-Whitney U-test. The relationship between BNMN and use of protective measures was evaluated using regression analysis.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable for glyphosate**

Comment: MN-test comparable to OECD guidelines, but not equal. Exposures to multiple pesticides with no information on exposure concentrations to individual pesticides make resulte unreliable for glyphosate.

2. Relevance of study: **Not relevant** (Due to the exposure of multiple pesticides, only general conclusions about pesticide exposure and cytogenicity possible. Not relevant to glyphosate).

3. Klimisch code: **3**

Author(s)	Year	Study title
Bolognesi, C., Landini, E., Perrone, E., Roggieri, P.	2004	Cytogenetic biomonitoring of a floriculturist population in Italy: micronucleus analysis by fluorescence in situ hybridization (FISH) with an all-chromosome centrometric probe Mutation Research Volume: 557 Number: 2 Pages: 109-117

Abstract*

Flower production in greenhouses associated with a heavy use of pesticides is very wide-spread in the western part of the Ligurian region (Italy). The formation of micronuclei in peripheral blood lymphocytes is a valuable cytogenetic biomarker in human populations occupationally exposed to genotoxic compounds. In the present study we investigated the micronucleus frequency in peripheral blood lymphocytes of 52 floriculturists and 24 control subjects by use of the cytokinesis-block methodology associated with fluorescence in situ hybridization with a pan-centromeric probe that allowed to distinguish centromere-positive (C+) and centromere-negative (C−) micronuclei. The comparison between floriculturists and controls did not reveal any statistically significant difference in micronucleus frequency, although an increase was observed with increasing pesticide use, number of genotoxic pesticides used and duration of exposure. An increase in C+ as well as in C− micronuclei and in the percentage of C+ micronuclei with respect to the total number of micronuclei was detected in floriculturists, suggesting a higher contribution of C+ micronuclei in the total number scored. The percentage C+ micronuclei was not related to the duration of exposure or to the number of genotoxic pesticides used, but a higher percentage (66.52% versus 63.78%) was observed in a subgroup of subjects using benzimidazolic compounds, compared with the floriculturist population exposed to a complex pesticide mixture not including benzimidazolics. These results suggest a potential human hazard associated with the exposure to this class of aneuploidy-inducing carcinogens.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	≥ 50 pesticides
Active substance(s):	≥ 50, including glyphosate
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Age of test persons:	Exposed group: 50.16 ± 13.67 y; Non-exposed: 46.83±10.59 y
Sex:	Exposed group: 44 male, 7 females non-exposed group: 15 males, 9 females
Smoking habits:	Exposed group: 11 smokers, 11 former smokers, 29 non-

	smokers Non-exposed group: 11 smokers, 5 former smokers, 8 non-smokers
Persons per group:	51 exposed; 24 non-exposed (control)
Exposure duration:	2-10 years (mean 26.34 ± 14.46)

4. Test system:

Study type:	Epidemilogical study for cytogenicity – Micronucleus assay + FISH
Guideline:	None
GLP / GCP:	No
Guideline deviations:	Not applicable
Application rate:	Not specified.
Persons per group:	51 exposed; 24 non-exposed (control)
Application technique:	Not specified
Mixing/loading performed:	Yes: 36 No: 15
Use of personal protective equipmet (PPE):	Yes: 44 No: 7
Cultivation conditions:	Greenhouses
Crops:	Ornamentals
Exposure conditions:	Not reported
Blood sampling:	Blood samples were obtained from each subject by venipuncture. All blood samples were collected in sterile sodium heparin tubes. The specimen were received in the laboratory within a few hours of collection and were processed immediately.
Cell cultures:	Whole blood was added to 4.5 mL of RPMI 1640 complete medium with 10 % FCS, and 1% phytohaemoagglutinin. Cells were cultured for 72 hours at 37°C, with cytochalasin B being added after 44 h (concentration: 6 µg/mL). At the end of the incubation period, whole blood cultures were centrifuged, washed, and cells were fixed twice in cold fixative (methanol : acetic acid 3:1) for 20 min at room temperature. Samples for microscopic evaluation were loaded onto wet slides, air dried and stained with 3% Giemsa or hybridized within 1 week of preparation
Fluorescence in situ hybridisation (FISH):	Centrometric FISH was performed using an alphoid centromer-specific biotinylated probe for all centromers, which was previously tested onmetaphase chromosome for centromer-specific labelling. Prepared slides were processed for hybridisation. The hybridisation mixture containing the probe (2.5 µg/mL) and 500 µg/mL salmon sperm DNA in 2 x SCC was denatured at 70°C for 5 min, followed by chilling on ice for 4 min. An aliquot of 50 µg per slide was applied. The slides were covered with coverslips and sealed with rubber cement. Hybridisation was performed for 16 h at 37°Y in a moist chamber. Afterwards, the slides were washed, and incubated with

blocking reagent (5% skimmed milk in 4 x SCC) at 37°C for 10 min. The slides were washed with 4 x SCC, covered with a 1.250 dilution of anti-biotin-antibody in IB (immunological buffer: 0.5% skimmed milk in 4 x SCC) and incubated at 37°C for 30 min. Afterward, slides were washed in and incubated in a 1:20-dilution of FITC-conjugated anti-mouse antibody, followed by incubation with a 1:20-dilution of FITC-conjugated anti-sheep antibody for 30 min at 37°C. All incubations were performed in a moist chamber, and were followed by washes in Tween-20 buffer. After the last wash, slides were dehydrated with ethanol and stained with propidium iodide in anti-dde solution.

5. Observations/analyses:

Questionnaire: All subjects. The following information was provided:

Demographic information, personal data, smoking habits, history of recent illness and medical treatment.

Exposed group: in addition, kind of crops handled, pesticide use, exposure duration, work activity, protective measures

Microscopic analyses: Giemsa stained slides:

2000 binucleated lymphocytes with preserved cytoplasm were scored for each subject. The MN frequency was calculated as the number of binucleated cells with micronuclei (BNMN).

FISH:

Slides were scored with a microscope with fluorescence equipment. The micronuclei present in the bi-nucleated lymphocytes with intact cytoplasm were examined for the presence of one or more centrometric spots and were classified as centromer-positive (C+MN) or centromer-negative (C – MN). 2000 binucleated lymphocytes with preserved cytoplasm were scored for each subject

Statistics. Parametric and non-parametric statistical test were used. Student´s t-test for independent samples was applied to detect differences in the mean of BNMN in the exposed and non-exposed subjects. Differences among the group means and between the percentages of C + MN and C – MN analysed by FIH technique were evaluated by non-parametric Mann-Whitney U-test. The relationship between C + MN and age was evaluated using regression analysis. The level of significance was taken as $p \leq 0.05$.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable for glyphosate**

Comment: Well-documented study. MN-test comparable to OECD guidelines, but not equal. No information on exposure concentrations to individual pesticides

2. Relevance of study: **Not relevant** (Due to the exposure of multiple pesticides, only general conclusions about pesticide exposure and cytogenic non-statistically significant differences possible. No statistically relevant findings reported for glyphosate alone).

3. Klimisch code: **2**

Author(s)	Year	Study title
Cavas, T., Könen S.	2007	Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (Carassius auratus) exposed to a glyphosate formulation using the micronucleus test and the comet assay Mutagenesis 22 263-268

Abstract*

Glyphosate is a widely used broad-spectrum weed control agent. In the present study, an in vivo study on the genotoxic effects of a technical herbicide (Roundup®) containing isopropylamine salt of glyphosate was carried out on freshwater goldfish Carassius auratus. The fish were exposed to three doses of glyphosate formulation (5, 10 and 15 ppm). Cyclophosphamide at a single dose of 5 mg/l was used as positive control. Analysis of micronuclei, nuclear abnormalities and DNA damage were performed on peripheral erythrocytes sampled at intervals of 48, 96 and 144 h post treatment. Our results revealed significant dose-dependent increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks. Our findings also confirmed that the alkaline comet assay and nuclear deformations in addition to micronucleus test on fish erythrocytes in vivo are useful tools in determining the potential genotoxicity of commercial herbicides.

* Quoted from article

MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Test item:	Roundup®
Active substance:	Glyphosate
Source:	Not reported
Lot/Batch #:	Not reported
Purity:	480 g/L isopropylammonium salt (equivalent to 360 g/L glyphosate)
Stability of test compound:	Not reported

2. Vehicle and/or positive control: Specified under the respective tests

3. Test animals:

Species:	Goldfish, *C. auratus*
Strain:	Linneaus, 1758
Family:	*Cyprinidae*
Source:	Local market
Age:	Not specified
Length:	6 ± 1 cm
Weight:	5 ± 1 g
Acclimation period:	3 weeks
Conditions:	At a population density of 15 per 20 L aquaria
Diet/Food:	Once per day with commercial fish pellets. Amount not specified.

Environmental conditions:	Temperature: 25°C 12 hours light/dark cycle

4. Test methods:

Micronucleus test (MNT):	Assessment of cytogenicity
Comet assay:	Assessment of cytogenicity, DNA damage
Guideline:	Not stated
GLP:	No
Guideline deviations:	Not applicable
Exposure conditions:	Goldfish were placed in four different aquaria containing declorinated tap water and three different concentrations of Roundup®, corresponding to 5, 10 and 15 ppm glyphosate obtained by serial dilutions of Roundup®. The test water was renewed every 2 days.
Exposure duration:	2, 4, or 6 days
Dose levels:	0, 5, 10, 15 ppm glyphosate
Negative control:	Tap water
Positive control:	Cyclophosphamid (5 mg/L)
Group size:	5 fish per dose per duration
Blood sampling:	At the end of each exposure period fish were killed by cervical dislocation. Blood samples were obtained from the caudal vein of the fish.
Sample processing and slide preparation:	For the MNT blood smears were prepared immediately after sampling onto pre-cleaned slides. After fixation in pure ethanol for 20 min, slides were allowed to dry and stained with 10 % Giemsa for 25 min. All slides were coded and scored blind. Five slides were prepared for each fish, and 1500 cells were scored from each slide. For the Comet assay, about 0.5 mL of blood was diluted with 1 mL of phosphate-buffered saline. The Comet assay was performed according to *Tice et al, 2000* [*Env. Mol. Mutagen.*, 35, 206-222] with some modifications. Electrophoresis conditions were: 0.66V/cm, 300 mM, for 25 min. Slides were neutralised and stained with ethidium-bromide and evaluated using a fluorescence microscope. From each fish five slides were prepared and from each slide 200 cells were scored.

5. Observations/analyses:

Measurements:	MNT: Non-refractive, circular or ovoid chromatin bodies, smaller than the one-third of the main nucleus, were scored as micronuclei. Nuclear abnormalities (NA) other than micronuclei in erythrocytes were classified into 5 groups: binucleated cells, blebbed nuclei, lobed nuclei, notched nuclei

Comet assay: DNA-damage was quantified by visual classification of cells into five categories (comets) corresponding to the tail length:

Type 0: undamaged
Type 1: low-level damage
Type 2: medium-level damage
Type 3: high-level damage
Type 4: complete damage
The extent of DNA damage was expressed as the mean % of cells with medium, high ad complete damageg DNA, which was calculated as the sum of cells with damage types 2, 3, 4. From the arbitrary values assigned to the different categories, a genetic damage index (GDI) was calculated for each fish.

Statistics: After assessing the normality of distribution of the data, both parametric and non-parametric tests were used to detect the level of significance at the 0.05 level. Differences between mean values were compared using the Student´s t-test and least significant difference test for the micronuclei data and the Mann-Whitney U-test for the Comet assay data.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Methodological and reporting deficiencies (e.g. test substance source, no concurrent measurement of toxicity reported, less than 2000 erythrocytes scored per animal and results not reported separately for replicates).

2. Relevance of study: **Relevant with restrictions** (Due to reliability. Discussion confuses glyphosate with glyphosate formulated products)

3. Klimisch code: **3**

Author(s)	Year	Study title
Guilherme, S. Gaivao, I. Santos, M.A. Pacheco, M.	2010	European eel (*Anguilla Anguilla*) genotoxic and pro-oxidant responses following short-term exposure to Roundup® - a glyphosate-based herbicide. Mutagenesis Volume: 25 Number: 5 Pages: 523-530

Abstract*

The glyphosate-based herbicide, Roundup®, is among the most used pesticides worldwide. Due to its extensive use, it has been widely detected in aquatic ecosystems representing a potential threat to non-target organisms, including fish. Despite the negative impact of this commercial formulation in fish, as described in literature, the scarcity of studies assessing its genotoxicity and underlying mechanisms is evident. Therefore, as a novel approach, this study evaluated the genotoxic potential of Roundup® to blood cells of the European eel (Anguilla anguilla) following short-term (1 and 3 days) exposure to environmentally realistic concentrations (58 and 116 *mg*/l), addressing also the possible association with oxidative stress. Thus, comet and erythrocytic nuclear abnormalities (ENAs) assays were adopted, as genotoxic end points, reflecting different types of genetic damage. The prooxidant state was assessed through enzymatic (catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase) and non-enzymatic (total glutathione content) antioxidants, as well as by lipid peroxidation (LPO) measurements. The Roundup® potential to induce DNA strand breaks for both concentrations was demonstrated by the comet assay. The induction of chromosome breakage and/or segregational abnormalities was also demonstrated through the ENA assay, though only after
3-day exposure to both tested concentrations. In addition, the two genotoxic indicators were positively correlated. Antioxidant defences were unresponsive to Roundup®. LPO levels increased only for the high concentration after the first day of exposure, indicating that oxidative stress caused by this agrochemical in blood was not severe. Overall results suggested that both DNA damaging effects induced by Roundup® are not directly related with an increased pro-oxidant state. Moreover, it was demonstrated that environmentally relevant concentrations of Roundup® can pose a health risk for fish populations.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup®
Active substance(s):	Glyphosate
Source of test items:	Bayer CropScience Portugal
Lot/Batch #:	Not reported
Purity:	485 g/L isopropylammonium salt of glyphosate (equivalent to 360 g/L or 30.8% of glyphosate)

2. Vehicle and/or positive control: No positive control

3. Test organism:

Species:	European eel (A. anguilla L.)
Source:	Captured from an unpolluted area of Aveiro Lagoon-Murtosa, Portugal
Length:	Average 25 ± 3 cm

Body weight:	32 ± 5 g (yellow eel stage)
Acclimation period:	12 days
Diet/Food:	Not fed during experimental period
Maintenance conditions:	During acclimatisation eels were kept in 80 L aquaria under a natural photoperiod, in aerated, filtered, dechlorinated and recirculated tap water
Physicochemical conditions of water:	Salinity: 0; Temperature: 20 ± 1 C; pH: 7.3 ± 0.2; Ammonia: <0.1 mg/L; Dissolved oxygen: 8.1 ± 0.5 mg/L

4. Test methods:

Comet assay:	Assessment of DNA strand breaks and alkali labelled sites
ENA assay:	Detection of micronuclei and other nuclear anomalies, clastogenicity, and aneugenicity
Catalase (CAT) activity:	Indicator of pro-oxidant state
Glutathion-S-transferase (GST) activity:	Indicator of pro-oxidant state
Indicator of pro-oxidant state Glutathion- peroxidise (GPx) activity:	Indicator of pro-oxidant state
Glutathion-reductase (GR) activity:	Indicator of pro-oxidant state
Total glutathion content (GSHt)-quantification:	Indicator of pro-oxidant state
Thiobarbituric acid reactive substances (TBARS) quantification:	Estimation for lipid peroxidation
Guideline:	None. Comet assay was done according to Collins (2004), Mol. Biotechnol. 26, 249-261M; with slight modifications
GLP:	No
Guideline deviations:	Not applicable
Dose levels:	58 µg/L (2 aquaria) and 116 µg/L (2 aquaria) of Roundup® (equivalent to 18 and 36 µg/L of glyphosate, respectively); 2 aquaria as controls with clean water.
Exposure duration:	For each pesticide concentration, 1- and 3-day exposures were tested, corresponding to the two different aquaria
Animals per dose group	36 eels divided in 6 aquaria
Exposure conditions:	The test was carried out in 20 L aquaria, in a static mode, under the same conditions as described for the acclimation period.
Blood sampling:	At the end of the exposure period blood was sampled from the posterior cardinal vein. Blood smears were immediately prepared for ENA assay. 2 µL of blood were diluted in 1 mL of phosphate-buffered saline for comet assay.. The remainder volume was stored at -80°C until further analyses for oxidative stress.
Tissue preparation and fractioning:	Whole-blood samples were lysed through homogenisation in a 1:15 ratio (blood : buffer, v/v), using a homogeniser in chilled phosphate buffer (0.2 M, pH 7.4). The lysate was divided into 3

aliquots for TBARS, GSHt quantification, as well as for post-mitochoindrial supernant (PMS) preparation. The PMS fraction was obtained by centrifugation (13400 g, 20 min, 4°C).

Test conditions: Comet assay: Two gel replicates each containing ca. 2 x 10^4 cells (using the blood samples) in 70 µL of 1% low melting point agarose in phosphate-buffered saline, were placed on glass mmicroscope slide, precoated with 1 % normal melting point agarose. Gels were covered with glass coverslips, left for ± 5 min at 4°C to sodify agarose and then immersed in lysis solution (2.5 M NaCl, 0.1 M ethylenediaminetetraacetic acid, 10 mM tris and 1 % Triton X-100, pH 10) at 4°C, for 1 h. Slides were immediately processed according to the conventional comet assay.

ENA assay:
This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos.

Blood smear per animal was fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min.

Replicates per dose level: Comet assay: 2

5. Observations/analyses:

Measurements: Comet assay: One slide with 2 gels (100 nucleotids/gel) was observed for each fish.
The DNA damage was quantified by visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail). The total score expressed as a genetic damage index (GDI) was calculated multiplying the mean percentage of nucleoids in each class by the corresponding factor.

Results were expressed as 'arbitrary units' in a scale of 0–400 per 100 scored nucleoids (as average value for the two gels observed per fish). Besides the GDI, the frequency of nucleoids observed in each comet class was also expressed as recommended by Azqueta et al.

ENA assay: From each smear 1000 erythrocytes were scored to determine the frequency of the following nuclear lesion categories: kidney shaped nuclei, lobed nuclei, binucleate or segmented nuclei, miclonuclei, and notched nuclei.

CAT activity: CAT activity was assayed (at 25 C) by the method of Claiborne as described by Giri et al. Change in absorbance was recorded spectrophotometrically at 240 nm and CAT activity was calculated in terms of micromoles H_2O_2 consumed per minute per milligram of protein (ε = 43.5/M cm).

GST activity: GST activity was determined (at 25 C) using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, according to the method of Habig et al. Absorbance was recorded spectrophotometrically at 340 nm for 3 min. The enzyme activity was calculated as nanomoles CDNB conjugate formed per minute per milligram of protein (ε = 9.6/mM cm).

GPx activity: GPx activity was determined (at 25 C) according

to the method of Mohandas et al. NADPH oxidation was recorded spectrophotometrically at 340 nm and GPx activity was calculated in terms of nanomoles NADPH oxidised per minute per milligram of protein ($\varepsilon = 6.22 \times 10^3/M\ cm$).

GR activity: GR activity was assayed (at 25 C) by the method of Cribb et al. The assay determines indirectly the GR activity by measuring the NADPH disappearance associated to the reduction of oxidised glutathione catalysed by GR. Change in absorbance was registered spectrophotometrically at 340 nm during 3 min and GR activity calculated as nanomoles of NADPH oxidised per minute per milligram of protein ($\varepsilon = 6.22 \times 10^3/M\ cm$).

GSHt quantification: For GSHt quantification – the rate of TNB production is proportional to the concentration of glutathione in the sample. Formation of TNB was measured by spectrophotometry at 412 nm and the results expressed as nanomoles TNB formed per minute per milligram of protein ($\varepsilon = 14.1/mM\ cm$).

TBARS quantification: As estimation of lipid peroxidation (LPO), TBARS quantification was carried out in the previously prepared lysate as adapted by Filho et al. The absorbance was measured at 535 nm and the rate of LPO was expressed in nanomoles of TBARS formed per milligram of fresh tissue ($\varepsilon = 1.56 \times 10^5/M\ cm$).

Total protein: Total protein contents were determined according to the Biuret method, using bovine serum albumin as a standard

Statistics: SigmaStat software (SPSS Inc.) was used for statistical analyses. All data were first tested for normality and homogeneity of variance to meet statistical demands. One-way analysis of variance was used to compare the different treatments within the same exposure duration as well as to compare the same treatment in different exposure durations. The Tukey's test was applied for post-hoc comparison. Whenever the assumptions for parametric statistics failed, the non-parametric correspondent test (Kruskall–Wallis) was performed, followed by the non-parametric all pairwise multiple comparison procedure (Dunn's test). Differences between means were considered significant when $P<0.05$. The relationship between the assessed parameters was explored using linear regression analyses. The correlation coefficient (r) was calculated and its statistical significance (P) was determined from the table of critical values for the correlation coefficient.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: No positive controls were included, which significantly detracts from the utility of a non-validated, non-standard test method. Less than the standard of a minimum of three dose levels used, independent coding of slides for scoring and results not reported separately for replicates.

2. Relevance of study: **Not Relevant** (Non-standard test system, no positive controls to verify test method/study validity.)

3. Klimisch code: **3**

Annex point	Author(s)	Year	Study title
IIA 5.10	Kale, P.G. Petty, B.T. Jr. Walker, S. Ford, J.B. Dehkordi, N. Tarasia, S. Tasie, B.O. Kale, R. Sohni, Y.R.	1995	Mutagenicity Testing of Nine Herbicides and Pesticides Currently Used in Agriculture. Environmental and Molecular Mutagenesis Volume: 25 Pages: 148-153

Abstract*

Nine herbicides and pesticides were tested for their mutagenicity using the Drosophila sex-linked recessive lethal mutation assay. These are Ambush, Treflan, Blazer, Roundup, 2,4-D Amine, Crossbow, Galecron, Pramitol, and Pondmaster. All of these are in wide use at present. Unlike adult feeding and injection assays, the larvae were allowed to grow in medium with the test chemical, thereby providing long and chronic exposure to the sensitive and dividing diploid cells, i.e., mitotically active spermatogonia and sensitive spermatocytes. All chemicals induced significant numbers of mutations in at least one of the cell types tested. Some of these compounds were found to be negative in earlier studies. An explanation for the difference in results is provided. It is probable that different germ cell stages and treatment regimens are suitable for different types of chemicals. Larval treatment may still be valuable and can complement adult treatment in environmental mutagen testing.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Pesticides: Ambush and Galecron; Herbicides: Treflan, Blazer, Roundup, 2,4-D Amine, Crossbow, Pramitol, Pondmaster
Active substance(s):	Ambush – permethrin Galecron – chlordimeform Treflan – trifluralin Blazer – scifluorfen Roundup – glyphosate 2,4-D Amine – 2,4-(Dichlorophenoxy) acetic acid Crossbow – 2,4,5-(Trichlorophenoxy) acetic acid Pramitol – prometon Pondmaster – glyphosate
Source:	Madison County Co-Op, Huntsville, Alabama
Lot/Batch #:	Not reported
Purity:	Not reported

Positive Contol	No positive control
2. Vehicle:	Distilled water
3. Test animals:	
Species:	Drosophila
Strains:	*Basc* genotype (females) and Canton-S (males, as a wild type)
Source:	Department of Biology, Alabama A. & M. University, Normal
Diet/Food:	Food pan was kept in the cage for 6 to 12 h
Collection of eggs:	The pan with eggs was kept at 25 °C for 24 h; Larvae were collected by adding 15% NaCl to the pan and then decanting the solution into a separatory funnel. The larvae were washed down with distilled water and collected on a piece of nylon gauze.
4. Test system:	
Study type:	Drosophila sex-linked recessive lethal (SLRL) test
Guideline:	Similar to, but not adhering to OECD 477
Guideline deviations:	No reference substances used; wild type male treatment age (treatment of larvae).
GLP:	No
Duration of study:	Not reported
Dose levels:	Control – only reported for Ambush – 0.1 ppm; Treflan – 1000 ppm; Blazer – 10000 ppm; Roundup – 1 ppm; 2,4-D – 10000 ppm; Crossbow – 10000 ppm; Galecron – 10000 ppm; Pramitol – 1000 ppm; Pondmaster – 0.1 ppm.
Treatment period:	Larval stage until the pupation
Treatment:	5 mL of each dilution was added to 3-4 mL of instant medium in vials. Each dilution was in sets with at least three vials. Larvae were added to the vials which were maintained in the hood at 25 C. In the control set of vials, 5 mL distilled water was added instead of the test chemical. Experiments were performed at LC_{50} except for very toxic compounds, where even lower survival points were used.
Reproduction procedure:	1 day old adult males treated as larvae were individually mated to six *Basc* virgins for 2 days to obtain the first brood. The males were then separated and each male was provided with a second set of virgins for the second brood. This procedure was repeated to obtain 6 consecutive broods

from individual males.

Inseminated females were allowed to lay eggs for 6 days and then discarded.

F1 progeny from each male were pair-mated separately in all broods.

Pair-matings were scored from 15 to 18 days after culturing.

The absence of wild-type males was used as the criterion for determining the lethal mutation.

Control experiments were performed similarly with untreated Canton-S males and virgin *Basc* females in order to determine the spontaneous mutation frequency.

5. Observations/analyses:

Measurements: Interbrood variations of sex-linked recessive lethal mutations;
Induction of sex-linked recessive lethals

Statistics: Simple χ^2 statistic was used

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Comparable to 1984 OECD guideline, but with several deficiencies (no positive controls reported and thus study validity not verifiable; wild type male treatment age different than recommended, purity of test substances not reported, tested formulation other ingredients such as surfactants not reported.)

2. Relevance of study: **Not Relevant to glyposate** (Glyphosate not tested; formulation tested)

3. Klimisch code: **3**

Annex point	Author(s)	Year	Study title
IIA 5.10	Manas, F. Peralta, L. Raviolo, J. Garcia Ovando, H. Weyers, A. Ugnia, L. Gonzalez Cid, M. Larripa, I. Gorla, N.	2009	Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests. Ecotoxicology and Environmental Safety Volume: 72 Pages: 834-837

Abstract*

Formulations containing glyphosate are the most widely used herbicides in the world. AMPA is the major environmental breakdown product of glyphosate. The purpose of this study is to evaluate the in vitro genotoxicity of AMPA using the Comet assay in Hep-2 cells after 4h of incubation and the chromosome aberration (CA) test in human lymphocytes after 48 h of exposition. Potential in vivo genotoxicity was evaluated through the micronucleus test in mice. In the Comet assay, the level of DNA damage in exposed cells at 2.5-7.5 mM showed a significant increase compared with the control group. In human lymphocytes we found statistically significant clastogenic effect AMPA at 1.8 mM compared with the control group. In vivo, the micronucleus test rendered significant statistical increases at 200-400 mg/kg. AMPA was genotoxic in the three performed tests. Very scarce data are available about AMPA potential genotoxicity.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	AMPA (aminomethylphosphonic acid)
Active substance(s):	AMPA (aminomethylphosphonic acid)
CAS-No.:	1066-51-9
Source:	Sigma-Aldrich, Argentina
Lot / Batch #:	Not reported
Purity:	Analytical grade. Not further specified.

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Cell lines:	Hep-2
Source:	Asociación Banco Argentino de Células (ABAC, Pergamino, Argentina)
Maintenance medium:	EMEM, supplemented with 200 mM L-glutamine, 2% (v/v) fetal calf serum, and antibiotics (penicillin, 100 U/ml, streptomycin 100 and 0.25 mg/ml anfotericine B)
Plate cultures:	96-well tissue culture plate (TPP® Zellkultur testplate 96F Switzerland) (8.25 x 10^5 cells/ml; 200 ml/well)
Culture conditions:	Cells were grown during 24 h at 37°C in an atmosphere of 5% CO_2 in air with 100% humidity to obtain confluent monolayers. Afterwards medium was removed and wells were replenished with MEM.

Primary cell culture:	Human lymphocytes
Source:	Six healthy donors, three females and three males, from 18 to 33 years old. The donors had no history of pesticide exposure and were non-smokers.
Culture conditions:	Lymphocytes were cultured for 72 h at 37 °C according to conventional methods.
Animals:	
Species:	Mice
Strain:	Balb-c
Source:	Not reported
Age at dosing:	8-12 weeks
Sex:	Males and females
Number of animals/group:	5
Weight at dosing:	Not reported
Acclimation period:	Not reported
Diet/Food:	Rodent diet, *ad libitum*
Water:	Water, *ad libitum*
Housing:	Not reported
Environmental conditions:	Not reported

4. Test methods:

GLP:	No (for all tests)
Single-cell gel electrophoresis assay (Comet assay):	Assessment of DNA damage
Guideline:	Non-guideline study. Study carried out according to Singh et al. (1988). (Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 84–191.)
Guideline deviations:	Not applicable, but slight modifications from Singh et al., 1988 regarding sample preparations.
Dose levels:	2.5, 4.5, 5.5, 6.5, 7.5, 9 and 10.0 mM AMPA
Positive control:	Mitomycin C, 0.01 mM
Negative control:	MEM medium
Conduct of test:	The test was conducted in 96-well tissue culture plates. In all cases the cultures were diluted to 200 µL/well final volume.
Exposure duration	4 h
Replicates per dose level:	2
Chromosome aberration (CA) test:	Assessment of cytogenicity
Guideline:	OECD 473 (1997) cited
Guideline deviations:	Lower number of used analysable concentrations, lower number of metaphases analysed.
Dose levels:	0.9 and 1.8 mM AMPA. The test substance was previously solved in 1 mL of culture medium and adjusted to pH 7.2-7.4.

Positive control:	Mitomycin C, 0.9 µM
Negative control:	Culture medium
Exposure duration:	Last 48 h of the culture duration of 72 h
Replicates per dose level:	2
Number of cells analysed	2000/replicate
***In vivo* Micronucleus test (MNT):**	Assessment of chromosome damage
Guideline:	OECD 474 (1997)
Guideline deviations:	Higher number of immature erythrocytes per animal scored for the incidence of micronucleated immature erythrocytes
Dose levels:	100, 200 mg/kg bw
Positive control:	Cyclophosphamid 20 mg/kg bw
Negative control:	Saline solution
Animals per dose group:	5
Exposure route:	i.p. injections at 24 h intervals
Number of treatments:	2 (test substance groups); 1 (control groups)
Sacrifice:	24 h after the second injection
Sampling and sample processing:	Bone marrow smears were prepared from the femoral bones. Smears were stained with May Grunwald Giemsa as described by Schmid (1975)

5. Observations/analyses:

Comet assay	
Measurements:	Cell viability (by tryptan blue exclusion technique), tail moment (TM), DNA percentage in tail (% of DNA) and tail length (TL)
Chromosome aberration-test	
Measurements:	Mitotic index determined for 2000 cells/replicate Chromosome aberrations: 100 metaphase cells were analysed for chromosome aberrations and classified into the following categories: Chromatid breaks (ctb) and gaps (ctg), chromosome breaks (csb) and gaps (csg), dicentric chromosomes (dic), acentric fragments (ace) and endoreduplicated (end) cells
***In vivo* MNT**	
Mortality/clinical signs:	Not reported
Measurements:	In order to score micronuclei about 1000 erythrocytes per animal were analysed. To evaluate bone marrow toxicity, 500 erythrocytes were counted and the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated
Statistics for all tests:	The mean scores were calculated from the experiments of each duplicate treatment. The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution. The non-parametric Kruskal–Wallis Analysis of Variance on Ranks ($p<0.05$) test followed by the Dunn's Multiple Comparisons Test were used for comparing the means of each treatment with their negative and positive control in the

Comet assessment. The Pearson statistical test was used to examine possible dose–response effects. In all cases, the level of significance was set at α=0.05.

KLIMISCH EVALUATION

1. Reliability of study:		**Not reliable**
	Comment:	Reporting deficiencies (purity of AMPA not specified, several parameters in the MNT not reported, only 2 dose levels used in both CA and MNT). Exposure route used in the MNT is not relevant for human exposure. Methodological deficiencies (see guideline deviations)
2. Relevance of study:		**Not relevant** (Due to reliability)
3. Klimisch code:		**3**

Author(s)	Year	Study title
Manas, F. Peralta, L. Raviolo, J. Garcia Ovando, H. Weyers, A. Ugnia, L. Gonzalez Cid, M. Larripa, I. Gorla, N.	2009b	Genotoxicity of glyphosate assessed by the comet assay and cytogenic tests Environmental Toxicology and Pharmacology Volume: 28 Pages: 37-41

Abstract*

It was evaluated the genotoxicity of glyphosate which up to now has heterogeneous results. The comet assay was performed in Hep-2 cells. The level of DNA damage in the control group (5.42±1.83 arbitrary units) for tail moment (TM) measurements has shown a significant increase ($p < 0.01$) with glyphosate at a range concentration from 3.00 to 7.50mM. In the chromosome aberrations (CA) test in human lymphocytes the herbicide (0.20–6.00mM) showed no significant effects in comparison with the control group. In vivo, the micronucleus test (MNT)was evaluated in mice at three doses rendering statistical significant increases at 400 mg/kg (13.0±3.08 micronucleated erythrocytes/1000 cells, $p < 0.01$). In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. Thiobarbituric acid reactive substances (TBARs) levels, superoxide dismutase (SOD) and catalase (CAT) activities were quantified in their organs. The results showed an increase in these enzyme activities.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate [*N*-(phosphonomethyl) glycine]
Active substance(s):	Glyphosate [*N*-(phosphonomethyl) glycine]
CAS-No.:	1071-83-6
Source:	Sigma-Aldrich, Argentina
Purity:	96%

2. Vehicle and/or positive control: Specified under the respective assays (see below)

3. Test system / cells / animals:

Cell lines:	Hep-2
Source:	Not reported
Maintenance medium:	EMEM, supplemented with 200 mM L-glutamine, 2% (v/v) fetal calf serum, and antibiotics (penicillin, 100 U/ml, streptomycin 100 and 0.25 mg/ml anfotericine B)
Plate cultures:	96-well tissue culture plate (8.25 x 10^5 cells/ml; 200 ml/well)
Culture conditions:	Not reported
Primary cell culture:	Human lymphocytes
Source:	Six healthy donors, three females and three males, from 18 to 33 years old. The donors had no history of pesticide exposure and were non-smokers.
Culture conditions:	Lymphocytes were cultured for 72 h at 37 °C according to conventional methods.

Animals:	Detailed parameters for mice are given only for the *in vivo* MNT, not for the TBARs, SOD and CAT determinations
Species:	Mice
Strain:	Balb-c
Source:	Not reported
Age at dosing:	8-12 weeks
Sex:	Males and females
Number of animals/group:	5
Weight at dosing:	Not reported
Acclimation period:	Not reported
Diet/Food:	Rodent diet, *ad libitum*
Water:	Water, *ad libitum*
Housing:	Not reported
Environmental conditions:	Not reported

4. Test methods:

GLP:	No (for all tests)
Single-cell gel electrophoresis assay (Comet assay):	Assessment of DNA damage
Guideline:	Non-guideline study. Study carried out according to Singh et al. (1988). (Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 84–191.)
Guideline deviations:	Not applicable, but slight modifications from Singh et al., 1988 regarding sample preparations.
Dose levels:	3.00, 4.50, 6.00, 7.50, 9.00, 12.0 and 15.0 mM glyphosate
Positive control:	Mitomycin C, 0.01 mM
Negative control:	MEM medium
Conduct of test:	The test was conducted in 96-well tissue culture plates. In all cases the cultures were diluted to 200 µL/well final volume.
Exposure duration	4 h
Replicates per dose level:	2
Chromosome aberration (CA) test:	Assessment of cytogenicity
Guideline:	OECD 473 (1997)
Guideline deviations:	Lower number of metaphases analysed than required
Dose levels:	0.20, 1.20, 6.00 mM glyphosate. The test substance was previously solved in 1 mL of culture medium and adjusted to pH 7.2-7.4.
Positive control:	Mitomycin C, 0.89 µM
Negative control:	Culture medium
Exposure duration:	Last 48 h of the culture duration of 72 h
Replicates per dose level:	2
Number of cells analysed	2000/replicate

***In vivo* Micronucleus test (MNT):**	Assessment of chromosome damage
Guideline:	OECD 474 (1997)
Guideline deviations:	Higher number of immature erythrocytes per animal scored for the incidence of micronucleated immature erythrocytes
Dose levels:	50, 100, 200 mg/kg bw
Positive control:	Cyclophosphamid 20 mg/kg bw
Negative control:	Saline solution
Animals per dose group:	5
Exposure route:	i.p. injections at 24 h intervals
Number of treatments:	2 (test substance groups); 1 (control groups)
Sacrifice:	24 h after the second injection
Sampling and sample processing:	Bone marrow smears were prepared from the femoral bones. Smears were stained with May Grunwald Giemsa as described by Schmid (1975)
***In vivo* TBARs, SOD, and CAT assay:**	*In vivo* determination of oxidant markers TBARs: thiobarbituric acid reaction products, SOD: superoxide dismutase, CAT: catalase
Guideline:	No
Guideline deviations:	Not applicable
Dose levels:	400 mg/kg bw
Positive control:	none
Negative control:	Saline solution
Animals per dose group:	5
Exposure route:	i.p. injection
Number of treatments:	1
Sacrifice:	1 and 2 h after the injection by cervical dislocation
Sampling and sample processing:	Livers, kidneys, hearts and lungs were removed. Tissue homogenates (10%) were prepared in chilled 0.05 M potassium phosphate buffer, pH 7.4

5. Observations/analyses:

Comet assay	
Measurements:	Cell viability (by tryptan blue exclusion technique), tail moment (TM), DNA percentage in tail (% of DNA) and tail length (TL)
Chromosome aberration-test	
Measurements:	The slides were scored blind by two observers. Mitotic index determined for 2000 cells/replicate Chromosome aberrations: 100 metaphase cells were analysed for chromosome aberrations and classified into the following categories: Chromatid breaks (ctb) and gaps (ctg), chromosome breaks (csb) and gaps (csg), dicentric chromosomes (dic), acentric fragments (ace) and endoreduplicated (end) cells

***In vivo* MNT**	
Mortality/clinical signs:	assessed
Measurements:	In order to score micronuclei about 1000 erythrocytes per animal were analysed. To evaluate bone marrow toxicity, 500 erythrocytes were counted and the ratio polychromatic erythrocytes/normochromatic erythrocytes (PCE/NCE) was calculated
***In vivo* TBARs, SOD, and CAT assay**	TBARs concentrations, expressed as nmol of malondialdehyde (MDA)/g of tissue were measured spectrophotometrically at 532nm in liver and kidney homogenates. The concentrations were determined using standard curves of MDA. Superoxide dismutase activity was assayed spectrophotometrically in the supernatants of liver homogenates. One unit of enzymatic activity has been defined as the amount of enzyme which causes 50% inhibition of auto oxidation of epinephrine. Catalase activity was measured at 240 nm by the decomposition of the H_2O_2.
Statistics for all tests:	Oneway ANOVA and Dunnett as “a posteriori” test were used in all the experiments. The Pearson statistical test was used to examine possible dose-response effects. In all cases, the level of significance was set at α=0.05.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Guideline deviations and reporting deficiencies. Several parameters in the MNT not reported. Blind scoring reported for the CA but not MNT. Exposure route used in the MNT is not relevant for human exposure. (see guideline deviations). No indication of pH or osmolality control for the comet assay. Results not reported separately for replicates.
2. Relevance of study:	**Not relevant** (Due to guideline deviations and reporting deficiencies)
3. Klimisch code:	**3**

Annex point	Author(s)	Year	Study title
IIA 5.10	Mladinic, M. Berend, S. Vrdoljak, A.L. Kopjar, N. Radic, B. Zeljezic, D.	2009	Evaluation of Genome Damage and Its Relation to Oxidative Stress Induced by Glyphosate in Human Lymphocytes in Vitro Environmental and Molecular Mutagenesis Volume: 50 Number: 9 Pages: 800-807

Abstract*

In the present study we evaluated the genotoxic and oxidative potential of glyphosate on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure. Testing was done with and without metabolic activation (S9). Ferric-reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS) and the hOGG1 modified comet assay were used to measure glyphosate's oxidative potential and its impact on DNA. Genotoxicity was evaluated by alkaline comet and analysis of micronuclei and other nuclear instabilities applying centromere probes. The alkaline comet assay showed significantly increased tail length (20.39 µm) and intensity (2.19%) for 580 µg/mL, and increased tail intensity (1.88%) at 92.8 µg/mL, compared to control values of 18.15 µm for tail length and 1.14% for tail intensity. With S9, tail length was significantly increased for all concentrations tested: 3.5, 92.8, and 580 µg/mL. Using the hOGG1 comet assay, a significant increase in tail intensity was observed at 2.91 µg/mL with S9 and 580 µg/mL without S9. Without S9, the frequency of micronuclei, nuclear buds and nucleoplasmic bridges slightly increased at concentrations 3.5 µg/mL and higher. The presence of S9 significantly elevated the frequency of nuclear instabilities only for 580 µg/mL. FRAP values slightly increased only at 580 µg/mL regardless of metabolic activation, while TBARS values increased significantly. Since for any of the assays applied, no clear dose-dependent effect was observed, it indicates that glyphosate in concentrations relevant to human exposure do not pose significant health risk.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Glyphosate
Description:	Not reported
Source:	Supelco, Sigma, St. Louis, MO, US
Lot/Batch #:	Not reported
Purity:	98%

2. Vehicle and/or positive control: Yes

Vehicle control:	Standard physiological solution

3. Test group:

Species:	Human
Tissue:	Blood
Age of test animals at study initiation:	Not reported
Sex:	Male

Dose levels: 0.5, 2.91, 3.5, 92.8, and 580 µg/mL

Metabolic activation: With and without (human liver mix)

Positive controls: Without metabolic activation: ethyl methanesulfonate, 200 µg/mL

With metabolic activation: cyclophosphamide, 30 µg/mL

4. Observations/analyses:

Antioxidant capacity

Test system: Ferric-reducing ability of plasma (FRAP)

To assess the antioxidant capacity of plasma its ability to reduce Fe^{3+} to Fe^{2+} the FRAP assay was measured by the FRAP assay. Fe^{3+} to Fe^{2+} reduction results in the formation of a coloured Fe^{2+}-TPTZ-complex with absorbance at 593 nm.

Working FRAP reagent was prepared as required by mixing 20 mL acetate buffer, 2.0 mL 2,4,6-tri[2-pyridyl]-s-triatine (TPTZ) solution, 2.0 mL $FeCl_3$ solution and 2.4 m distilled water. 30 µL of centrifuged plasma sample diluted in saline (1:1) was then added to 1 mL of freshly prepared reagent warmed at 37 °C.

Water solutions of known $FeSO_4$ concentration, in the range of 0.1–1.0 mM, were used for obtaining the calibration curve. As a positive control, 0.5 mL whole blood was treated with vitamin C at a final concentration of 100 µg/mL.

Lipid peroxidation

Test system: Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid (TBA) reacts calorimetrically with malondialdehyde (MDA), a secondary product of lipid peroxidation and gives an index of the extent of lipid peroxidation.

5 µL 0.2% (w/v) butylated hydroxytoluene (BHT) and 750 µL 1% (v/v) phosphoric acid was added to 50 µL plasma sample. After mixing, 250 µL 0.6% (w/w) TBA and 445 µL H_2O were added and the reaction mixture was incubated in a water bath at 90 °C for 30 min. The mixture was cooled and absorbance was measured at 532 nm.

The concentration of TBARS was calculated using standard curves of increasing 1,1,3,3-tetramethoxypropane concentrations, and expressed as µmol/L.

Cell viability and necrosis

Test system: Vital Staining

The indices of cell viability and necrosis were obtained from differential staining with acridine orange and ethidium bromide, using fluorescence microscopy. Both dyes intercalate with the DNA, but acridine orange is cell-permeable in contrast to ethidium bromide.

50 µL of treated blood was mixed with the same amount of acridine orange (100 µg/mL) and ethidium bromide (100 µg/mL, 1:1; v/v). The suspension mixed with dye was covered with a cover slip and analyzed under the epifluorescence microscope.

400 lymphocytes were analyzed (200 per duplicate culture) for each lymphocyte culture (concentration), counting the unstained (viable) cells. The nuclei of vital cells emitted a green fluorescence; apoptotic lymphocytes emitted a green fluorescence surrounded by a red echo and necrotic cells red fluorescence.

DNA damage

Test system: Alkaline and hOGG1Modified Comet Assay

The comet assay measures DNA strand breaks by embedding cells in agarose and lysing the cells with detergent and high salt. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks.

Blood samples (8 µL) were mixed with 0.5% low melting point agarose, were immersed in freshly prepared ice-cold lysis solution (pH 10) and stored at 4 °C overnight. For the alkaline comet assay the standard procedure was followed.

In addition, the hOGG1Modified Comet Assay is able to infer the type of DNA damage from the substrate specificity of human 8-oxoguanine DNA glycosylase 1 (hOGG1). For this assay, the hOGG1 FLARE™ Assay Kit (Trevigen) was used.

Comet assay analysis was done in duplicates.

Chromosomal and Nuclear Instability

Test system: Fluorescence in situ hybridization (FISH)

Cultivation of lymphocytes gained from the blood samples and slide preparation was done according to standard protocol (Fenech, 2006).

Cytokinesis was arrested using cytochalasin B at a final concentration of 6 µg/mL and added to the culture after 44 hr of incubation. Cells were centrifuged, washed in 0.9% NaCl solution and fixed with 3:1 (v/v) methanol/acetic acid solution. Slides were stained with 5% Giemsa.

One thousand binucleated cells with well-preserved cytoplasm were scored per subject, to determine the total number of micronuclei in binucleated lymphocytes (MN), nuclear buds (NBs), and nucleoplasmic bridges (NPBs). The cytokinesis-block proliferation index (CBPI) was evaluated by classifying 1000 cells per number of nuclei, according to the formula: CBPI = [M1+2M2+3(M3+M4)]/N, where M1-M4 indicate the number of cells with 1–4 nuclei respectively, and N the total number of cells scored. To detect the ratio of micronuclei (C+MN), nuclear buds (C+NB), and nucleoplasmic bridges (C+NPB) originating from whole chromosomes that contain centromeres, and the number of DAPI signal positive micronuclei (+MN), slides were kept in dark for a month.

Slides were hybridized with All Human Centromere Satellite Probes directly labelled with a red fluorophore (Texas Red spectrum) following the supplier's instructions. Slides were counterstained with DAPI prepared in an antifade solution. 1000 binucleated lymphocytes were analyzed for each

concentration.

KLIMISCH EVALUATION

1. Reliability of study:		**Reliable with restrictions**
	Comment:	Non-GLP, non-guideline *in vitro* study, meeting scientific principles
2. Relevance of study:		Relevant with restrictions (Assessment of Genotoxicity *in vitro* at concentrations relevant to human exposure levels; authors state that no clear dose-dependent effect was observed, and results indicate that glyphosate in concentrations relevant to human exposure do not pose significant health risk.
3. Klimisch code:		**2**

Annex point	Author(s)	Year	Study title
IIA 5.10	Mladinic, M., Perkovic, P., Zeljezic, D.	2009b	Characterization of chromatin instabilities induced by glyphosate, terbuthylazine and carbofuran using cytome FISH assay Toxicology Letters Volume: 189 Number: 2 Pages: 130-137

Abstract*

Possible clastogenic and aneugenic effects of pesticides on human lymphocytes at concentrations likely to be encountered in residential and occupational exposure were evaluated with and without the use of metabolic activation (S9). To get a better insight into the content of micronuclei (MN) and other chromatin instabilities, lymphocyte preparations were hybridized using pancentromeric DNA probes. Frequency of the MN, nuclear buds (NB) and nucleoplasmic bridges (NPB) in cultures treated with glyphosate slightly increased from 3.5 µg/mL onward. Presence of S9 significantly elevated cytome assay parameters only at 580 µg/mL. No concentration-related increase of centromere (C+) and DAPI signals (DAPI+) was observed for glyphosate treatment. Terbuthylazine treatment showed a dose dependent increase in the number of MN without S9 significant at 0.0008 µg/mL and higher. At concentration lower than 1/16 LD50 occurrence of C + MN was significantly elevated regardless of S9, but not dose related, and in the presence of S9 only NBs containing centromere signals were observed. Carbofuran treatment showed concentration dependent increase in the number of MN. The frequency of C + MN was significant from 0.008 µg/mL onward regardless of S9. Results suggest that lower concentrations of glyphosate have no hazardous effects on DNA, while terbuthylazine and carbofuran revealed a predominant aneugenic potential.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test items:	Glyphosate, terbuthylazine, and carbofuran
Active substance(s):	Glyphosate, terbuthylazine, and carbofuran
Description:	Not reported
Source of test medium:	Supelco, Sigma, St. Louis, MO, USA
Lot/Batch #:	Not reported
Purity:	98%

2. Vehicle, negative and/or positive control:

Vehicle: PBS, pH 7.2

Positive control: ethyl methanesulfonate (-S9, 200 µg/mL) and cyclophosphamide (+S9, 30 µg/mL)

Negative control: Standard physiological solution

3. Test system / cells:

Cell cultures:	Lymphocytes
Species:	Human
Source:	Three young, healthy, non-smoking voluntary donors that were not exposed to any physical or chemical agent that could interfere the results 12 month prior to blood sampling.

Culture medium	RPMI 1640 medium without mitogen and newborn calf serum
Culture conditions:	Temperature: 37°C

4. Test method:

Study type:	FISH cytome analysis
Guideline:	Non-guideline study, but similar to OECD 487 with additional analysis
GLP:	No
Guideline deviations:	Not applicable
Test conditions:	Each culture was treated with glyphosate diluted in PBS. The test substance was tested with and without metabolic activation. After the treatment period samples were washed two times in 0.5 mL of culture medium and centrifuged. The supernatant was removed and pellet was used to set up cultures by adding it to 6 mL of culture medium supplemented with 15% foetal calf serum, and 1% antibiotics (penicillin and streptomycin). Then lymphocytes were stimulated by 1% phytohaemagglutinin and incubated for 72 h at 37°C. Cultivation of lymphocytes gained from the blood samples and slide preparation was done according to standard protocol (Fenech, 2006). Cytokinesis was arrested by means of cytochalasin B (6 µg/mL) added to the culture after 44 h incubation. Cells were centrifuged, washed in 0.9% NaCl solution and fixed with 3:1 (v/v) methanol/acetic acid solution. Slides were stained with 5% Giemsa.
Metabolic activation:	10% w/w of human liver S9 mix
Dose levels:	0.50, 2.91, 3.50, 92.8, and 580 µg/mL glyphosate
Exposure duration:	4 h (test samples) and 72 h (positive control cultures)
Replicates:	2 per human donor

5. Observations/analyses:

Measurements:	1000 binucleated cells with well-preserved cytoplasm were scored per subject for determination of the total number of micronuclei (MN), nuclear buds (NBs), and nucleoplasmic bridges (NPBs). The cytokinesis-block proliferation index (CBPI) was assessed by classifying 1000 cells per number of nuclei, according to the formula: CBPI = [M1+2M2+3(M3+M4)]/N, where M1-M4 designate the number of cells with 1–4 nuclei respectively, and N the total number of cells scored. Slides were kept in dark for a month for detection of the ratio of micronuclei (C+MN), nuclear buds (C+NB), and nucleoplasmic bridges (C+NPB) originating from whole chromosomes containing centromeres, and the number of DAPI signal positive MN (DAPI+MN) and DAPI signal positive NB (DAPI+NB). Slides were hybridized with All Human Centromere Satellite Probes directly labelled with a red fluorophore (Texas Red

spectrum) following the supplier's instructions. Slides were counterstained with DAPI prepared in an antifade solution. 1000 binucleated lymphocytes were analyzed for each concentration.

Ratio of centromere-positive micronuclei (C+MN) was calculated by dividing the number of MN containing the centromere signal with the total number of MN counted for the specific treatment. The same approach was used for calculating ratio of C+NB, and C+NPB. Ratio of DAPI signal positive micronuclei (DAPI+MN) was obtained by dividing the number of MN displaying intensive DAPI signal with total number of MN counted for specific treatment.

Statistics: Evaluation was done in triplicate. Results are presented as mean ± SD. Differences in the number of different parameters between treated and control cultures and with and without S9 were evaluated using the Fisher LSD test. The correlations between different measured parameters were analysed by means of Spearman correlation test.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Non-GLP, non-guideline study *in vitro*. Positive and negative control results almost indistinguishable for MN assay without metabolic activation. Negative control NB and NBP results not reported.

2. Relevance of study: **Not Relevant** (Proposed mechanism of genotoxicity (*in vitro*) is not relevant to human exposure levels. Authors express confidence that estimated maximum human exposure levels correspond to acceptable safety levels based on evaluated *in vitro* endpoints, and that their findings need to verified *in vivo*.)

3. Klimisch code: **3**

Author(s)	Year	Study title
Paz-Y-Mino, C. Sanchez, M. E. Arevalo, M. Munoz, M. J. Witte, T. De-La-Carrera, G. O. Leone, P. E.	2007	Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate. Genetics and Molecular Biology Volume: 30 Number: 2 Pages: 456-460

Abstract*

We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. A total of 24 exposed and 21 unexposed control individuals were investigated using the comet assay. The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5 mu m) compared to the control group (comet length = 25.94 mu m). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup Ultra®
Active substance(s):	Glyphosate
Description:	Not reported
Source of test item:	Not reported
Lot/Batch #:	Not reported
Purity:	43.9 % glyphosate

2. Vehicle and/or positive control: None

3. Test group:

Species:	Human
Age of test persons:	Exposed group: 17-59 y; non-exposed: 16-53 y
Sex:	Exposed group: 1 male, 23 females non-exposed group: 4 males, 17 females

4. Test system:

Study type:	Epidemilogical study for cytogenicity – Comet assay
Guideline:	Non
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	About 3 month
Application rate:	23.4 L product/ha (= 10.3 L glyphosate/ha); The application of the glyphosatew product was combined with the adjuvant “Cosmoflux 411F”, that increases the adherence or absorption of the herbicide. The concentration of the adjuvant in the spray solution is not specified.

Persons per group:	24 exposed; 21 non-exposed (control)
Application technique:	Aerial spraying
Test conditions:	The exposed group consisted of 24 randomly selected individuals who lived ≤ 3 km from an area where a glyphosate-based herbicide was applied. Exposure occurred on three consecutive days followed by sporadic aerial spraying over a three-week period. One-half of this group were exposed due to direct spray application over their houses; the other half lived within 200 m to 3 km from spray areas. The non-exposed group consisted of 21 healthy individuals living 80 km away from the spraying area. None of the persons (exposed, non-exposed) were involved in application of pesticides. Activities performed were mainly in the house and sometimes cultivation and harvesting.
Blood sampling:	Exposed group: venous blood (5 mL) was taken from the exposed individuals between 2 weeks and 2 month after their exposure and processed immediately after collection. Non-exposed group: Blood samples were collected and processed as for the exposed group, but not concomitantly.

5. Observations/analyses:

Clinical history:	Exposed persons only
Clinical signs:	Exposed persons only
Body weight:	All persons
Cytogenicity:	Comet assay with venous blood
Haematology:	Not performed.
Clinical chemistry	Not performed.
Urine analysis	Not performed.
Statistics.	Mann-Whitney U test was applied to determine the differences between exposed and non-exposed group in the comet assay.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Documentation of Comet assay insufficient for assessment.
2. Relevance of study:	**Not relevant** (Glyphosate formulation was applied at much higher dose rates than recommended for the intended uses in the EU. In addition, the herbicide was combined with the adjuvant (Cosmoflux 411F) that can increase the biological action of the herbicide. This adjuvant will not be used in the EU.)
3. Klimisch code:	3

Author(s)	Year	Study title
Peluso, M. Munnia, A. Bolognesi, C. Parodi, S.	1998	^{32}P-postlabeling detection of DNA adducts in mice treated with the herbicide Roundup. Environmental and Molecular Mutagenesis Volume: 31 Number: 4 Pages: 55-59

Abstract*

Roundup is a postemergence herbicide acting on the synthesis of amino acids and other important endogenous chemicals in plants. Roundup is commonly used in agriculture, forestry, and nurseries for the control or destruction of most herbaceous plants. The present study shows that Roundup is able to induce a dose-dependent formation of DNA adducts in the kidneys and liver of mice. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 +/- 0.1 (SE) and 1.7 +/- 0.1 (SE) adducts/10(8) nucleotides, respectively. The Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate, but to another, unknown component of the herbicide mixture. Additional experiments are needed to identify the chemical specie(s) of Roundup mixture involved in DNA adduct formation. Findings of this study may help to protect agricultural workers from health hazards and provide a basis for risk assessment.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup ®
Active substance(s):	Glyphosate (as isopropylammonium salt)
Source:	Monsanto, Milan, Italy
Purity:	30.4% isopropylammonium salt
Test item:	Formulated isopropylammonium salt of glyphosate with surfactant
Source:	Societa Italiana Chimici, Rome, Italy

2. Vehicle: Dimethylsulfoxide (DMSO)/ olive oil

3. Test animals:

Species:	Mice
Strain:	Swiss CD1
Source:	Charles River, Como, Italy
Age of test animals at study initiation:	8-10 weeks
Sex:	Males and females
Body weight:	Not reported
Acclimation period:	Not reported
Diet/Food:	Not reported
Water:	Not reported

Housing: Not reported

Environmental conditions: Not reported

4. Test system:

Study type: ^{32}P-postlabeling detection of DNA adducts in mice

Guideline: Non-guideline study

GLP: No

Guideline deviations: Not applicable

Duration of study: 24 h

Dose levels: Controls; and

Roundup: 400, 500, and 600 mg/kg bw, corresponding to 122, 152, and 182 mg/kg bw of glyphosate salt.

Isopropylammonium salt of glyphosate: 130 and 270 mg/kg bw.

Animals per dose group: DMSO/olive oil – 6 animals;

Roundup:
400 mg/kg (122 mg/kg of glyphosate salt) – 6 animals;
500 mg/kg (152 mg/kg of glyphosate salt) – 3 animals;
600 mg/kg (182 mg/kg of glyphosate salt) – 3 animals.

Isopropylammonium salt of glyphosate:
130 mg/kg – 6 animals;
270 mg/kg – 3 animals;

Route of exposure: Intra-peritoneal (i.p.)

5. Observations/analyses:

Test substance preparations: Stability, achieved concentrations, and homogeneity not reported

Sample preparation: Kidneys and liver were separately pooled and DNA was isolated by a procedure involving enzymatic digestion of protein and RNA and solvent extraction.

Measurements: ^{32}P-postlabeling. The level of DNA adducts was determined by excising areas of chromatograms and measuring the levels of radioactivity present by Cerenkov counting. Quantitation of normal nucleotides was carried out as previously described [Taningher et al., 1995].

Statistics: Not reported

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: A non-guideline study with confounding results based on testing a surfactant containing formulation. Reporting

	deficiencies (statistical methods). Toxic surfactant effects subsequently verified in Heydens et al. (2008) reporting the same study type with a glyphosate formulated product and an appropriate control; formulation blank without glyphosate.
2. Relevance of study:	**Not relevant** (i.p. administration of high doses of a surfactant containing formulation a relevant exposure scanario for human risk assessments. In addition, the DNA adducts observed were not related to the active ingredient (isopropylammonium salt of glyphosate), but to another, unknown component of the herbicide mixture.)
3. Klimisch code:	**3**

Author(s)	Year	Study title
Poletta, G.L. Larriera, A. Kleinsorge, E. Mudry, M.D.	2009	Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris*) evidenced by the Comet assay and Micronucleus test Mutation Research Volume: 672 Number: 2 Pages: 95-102

Abstract*

The genotoxicity of pesticides is an issue of worldwide concern. The present study was undertaken to evaluate the genotoxic potential of a widely used herbicide formulation, Roundup® (glyphosate), in erythrocytes of broad-snouted caiman (*Caiman latirostris*) after in ovo exposure. Caiman embryos were exposed at early embryonic stage to different sub-lethal concentrations of Roundup® (50, 100, 200, 300, 400, 500, 750, 1000, 1250 and 1750 µg/egg). At time of hatching, blood samples were obtained from each animal and two short-term tests, the Comet assay and the Micronucleus (MN) test, were performed on erythrocytes to assess DNA damage. A significant increase in DNA damage was observed at a concentration of 500 µg/egg or higher, compared to untreated control animals ($p<0.05$). Results from both the Comet assay and the MN test revealed a concentration-dependent effect. This study demonstrated adverse effects of Roundup® on DNA of *C. latirostris* and confirmed that the Comet assay and the MN test applied on caiman erythrocytes are useful tools in determining potential genotoxicity of pesticides. The identification of sentinel species as well as sensitive biomarkers among the natural biota is imperative to thoroughly evaluate genetic damage, which has significant consequences for short- and long-term survival of the natural species.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® Full
Active substance(s):	Glyphosate (as potassium salt)
Source:	Agroservicios Humboldt, Santa Fe, Argentina
Purity:	66.2% glyphosate
Lot/Batch #:	Not reported

2. Vehicle and/or positive control: Vehicle – distilled water;
Positive control – cyclophosphamide (CP)

3. Test system:

Species:	*Caiman latirostris*
Test system:	Eggs
Source:	Fisco field ($30^0$11'26''S; $61^0$0'27''W), Santa Fe Province, Argentina
Sex:	Not reported
No. of eggs:	Experiment #1 – 100 Experiment #2 - 84
Egg weight:	Experiment #1 – 67.5 ± 4.89 g;

Experiment #2 – 72.7 ± 7.21 g

Acclimation period: Not reported

Environmental conditions: All nests used in each experiment were collected within 5 days after oviposition, on the same day and maintained under the same conditions from harvest to treatment assignment.

4. Test methods:

Study type: Genotoxicity study: Comet assay, micronucleus assay

Duration of study: Not specified

Dose levels: <u>Experiment #1</u>
Control group – distilled water, 50 µL;
Positive control groups – CP, 700 or 1400 µg/egg;
Treatment groups – 50, 100, 200, 300, 400, 500 and 1000 µg/egg

<u>Experiment #2</u>
Control group – distilled water, 50 µL;
Positive control group – CP, 700 µg/egg;
Treatment groups – 500, 750, 1000, 1250 and 1750 µg/egg

Eggs per dose group: <u>Experiment #1</u>
10 groups of 10 eggs each (5 eggs per each, two replicas)
<u>Experiment #2</u>
7 groups of 12 eggs each (6 eggs per each, two replicas)

Administration: All treatments were presumably a single dose, applied topically to the eggshell dissolved in 50 µL of distilled water (CP and Roundup treatments). Applications were done at early embryonic stage, within the first 5 days after oviposition, based on the opaque eggshell banding development

Experimental conditions: Artificial incubator;
Temperature: 31 ± 1°C
Humidity: 95%
Peripheral blood samples (0.5 ml) were obtained from each hatchling from the spinal vein, with heparinized disposable syringes.

Test methods:

<u>Micronucleus test</u>

Guideline: OECD 474

GLP: No

Guideline deviations: Modified to be applied in *C. latirostris* erythrocytes; housing and feeding conditions of parents not specified; sex not distinguished.

1) Modification
The MN assay originally performed in peripheral blood lymphocytes was modified to be applied in *C. latirostris*: application on eggs (within 5 days after oviposition), blood sampling after hatching.

2) Preparation
Two smears were prepared from each animal, coded for 'blind' analysis and stained with Acridine Orange supravital stain at

the moment of analysis.

Comet assay

Guideline: Non- guideline

GLP: No

Guideline deviations: Modified to be applied in *C. latirostris*

1) Cell preparation
Cell viability was determined before the application of the SCGE by fluorescent DNA-binding dyes. The cell suspension was mixed with a dye-mix working solution of 100 µg/ml Acridine Orange and 100 µg/ml ethidium bromide, prepared in Ca^{2+} - and Mg^{2+} -free PBS and then examined under a fluorescent microscope (40×). A total of 100 cells were counted per sample and the percentage of viable cells was determined.

2) Electrophoresis
The alkaline Comet assay was performed as described by Singh et al. with modifications required by *C. latirostris* erythrocytes, determined in previous studies: blood samples were diluted 1:19 (v/v) with RPMI-1640 medium and 1.5µL of the dilution (4.0×10^3 erythrocytes, approximately) were used to prepare each of two slides per blood sample, following standard protocol. Slides were immersed in lysis buffer for 24 h, incubated in alkaline buffer for 10 min and electrophoresed at 300 mA and 25V (0.90 V/cm) during 10 min also.

To demonstrate the electrophoresis conditions, positive controls were included in each electrophoresis carried out. The result of each electrophoresis was considered only if the positive controls showed positive results.

5. Observations/analyses:

Test substance preparations: Stability, achieved concentrations, homogeneity not reported

Mortality: Not reported

Clinical signs: Not reported

Body weight: Measured (OHAUS® Compact scale CS200, precision 0.1)

Body length: Measured (tape measure, precision 0.5 cm)

Snout-vent length: Measured (tape measure, precision 0.5 cm)

Identification: Individually identified by two numbered webbing tags in the hindlegs (National Band and Tag Co., Newport, KY)

Food- and water consumptions: Not relevant

Haematology: Not reported

Clinical chemistry: Not reported

Urine analysis: Not reported

Sacrifice/pathology: Not reported

Organ weights: Not reported

Histology: Not reported

Measurements: Micronucleus test
Microscopy: The frequency of MN was manually scored using

a fluorescent microscope (Olympus CX 40) equipped with a U-RFLT 50 excitation filter.

For each individual, 1000 erythrocytes were analysed in two replicated slides and the frequencies of micronucleated cells among them were recorded.

Comet assay

Microscopy: All samples were coded and evaluated blindly. At the moment of analysis, the slides were stained with ethidium bromide (2µg/mL). Comet images were analyzed using the fluorescent microscope.

Statistics: Images of 100 randomly selected cells (50 cells from each of two replicated slides) were scored from each animal. Cells were visually classified into five classes according to tail size and intensity (from undamaged, class 0, to maximally damaged, class 4), resulting in a single DNA damage score (damage index, DI = $n1$+2 $n2$+3 $n3$+4 $n4$) for each animal. Mean values ± standard error of MN and DI were calculated from data of animals of each experimental group. Statistical analysis was performed using the software SPSS 14.0 for Windows. Variables were tested for normality with Kolmogorov–Smirnov test and homogeneity of variances between groups was verified by Levene test. One-way ANOVA followed by Dunnett's test was used for the comparison of MN frequencies and DI between each group exposed to Roundup or CP and the negative control. A difference of $p < 0.05$ was considered statistically significant. Linear regressions were carried out to determine the existence of a concentration-dependent effect of Roundup on DI and MN frequencies. Data from Experiment 1 and Experiment 2 groups (from Roundup 50 to Roundup 1750) were considered together, taking into account that experiment conditions were exactly the same for both experiments except for Roundup concentrations applied. Concentration-dependent analysis was performed on MN and DI total data as well as on MN and DI mean values of each Roundup experimental group.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable**

Comment: Non-GLP studies in a unique test model. Micronucleus assay followed guideline, Comet assay similar to guideline.

Test methods have been modified to be applied caiman species. Methodological deficiencies: housing and feeding conditions of parents not specified; sex not distinguished, stability and homogeneity assessment of test substance preparations not reported. Results not reported separately for replicate individual animals.

2. Relevance of study: **Not Relevant**. Highly artificial *in ovo* exposure scenario not relevant to real world environmental exposures. Caiman eggs are covered and not exposed to the surface. Any glyphosate in

	a potential herbicide overspray would sorb to sediment and organic matter and not transport to the egg surface.
3. Klimisch code:	**3**

Author(s)	Year	Study title
Rodrigues, H.G. Penha-Silva, N. Ferreira Pereira de Araujo, M. Nishijo, H. Aversi-Ferreira, T.A.	2011	Effects of Roundup® Pesticide on the Stability of Human Erythrocyte Membranes and Micronuclei Frequency in Bone Marrow Cells of Swiss Mice The Open Biology Journal Volume: 4 Pages: 54-59

Abstract*

Pesticides can affect the health of living organisms through different mechanisms such as membrane denaturation. The evaluation of the deleterious effects of chemical agents on biological membranes can be performed through the analysis of the stability of erythrocytes against a concentration gradient of certain chemical agent in physiologic saline solution. This work analyzed the effect of the herbicide Roundup® on the membrane of human erythrocytes in blood samples collected with EDTA or heparin as anticoagulant agent. The results were analyzed through spectrophotometry at 540 nm and light microscopy. There was an agreement between spectrophometric and morphologic analyses. At the concentration limit recommended for agricultural purposes, Roundup® promoted 100% of hemolysis. The D_{50}Roundup® values obtained for human blood samples collected with EDTA were not significantly different from those obtained for samples collected with heparin. However, the lysis curves presented lower absorbance values at 540 nm in the presence of blood collected with EDTA in relation to that collected with heparin, probably due to haemoglobin precipitation with EDTA. This work also analyzed the effects of three different Roundup® doses (0.148, 0.754 and 1.28 mg/kg) on the micronuclei frequency in bone marrow cells of Swiss mice in relation to a positive control of cyclophosphamide (250 mg/kg). The two highest Roundup® doses showed the same genotoxicity level as the positive control.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Roundup®
Active substance(s):	Glyphosate
Source:	Not reported
Purity:	Not reported

2. Vehicle and/or positive control: Not applicable

3. Test group:

Species:	Human
Tissue:	Blood
Number of test persons:	8
Age:	24 ± 3 years
Sex:	Not reported
Inclusion criteria:	o healthy o non-smokers o non-users of drugs or medications o non-consumers of alcoholic beverages

Species:	Mice
Strain:	Swiss
Source	Iquego, Goiânia, Goiás, Brazil
Number of animals:	Not reported
Age:	7-12 weeks
Sex:	Not reported
Environmental conditions:	o 26 ± 2 °C o 12 h light/dark cycle o *ad libitum* access to food and water

4. Test system:

Type:	Determination of the stability of human erythrocytes
Guideline:	Non-guideline study
GLP / GCP:	No
Dose levels:	*In physiologic saline solution:* 0 to 40 µL/100 mL Roundup® (40 µL/dL) in 0.9%NaCl, (concentration range within the acceptable limit for use of the herbicide in agriculture according to the manufacturer) *In aqueous solution:* 0 to 40 µL/100 mL Roundup® (40 µL/dL) as aqueous solution
Sample collection:	Blood samples (3 mL) were collected from volunteers through intravenous puncture after an 8-12 h fasting period. The blood collections were performed in evacuated tubes containing 50 µL of 25 mmol/L K_2EDTA or 50 µL of heparin as anticoagulant.

Type:	Micronuclei frequency in Mice
Guideline:	Non-guideline study
GLP / GCP:	No
Dose levels:	0, 0.148, 0.754 and 1.28 mg/kg bw
Negative control:	Saline solution
Positive control:	Cyclophosphamide; 250 mg/kg bw
Sample collection:	Drugs were prepared in 0.2 mL of sterile saline solution and intraperitoneally administrated. 24 h after treatment, the animals were sacrificed by cervical dislocation, and then the bone marrow of both femurs was collected for the preparation of slides. The bone marrow was collected with the aid of a 1 mL syringe filled with saline. The femur was washed with saline and cell suspension was collected in a test tube containing saline. The cell suspension was centrifuged for 5 minutes at 1300 g, discarding the supernatant and retaining a volume of 0.5 mL in the tube for later re-suspension and homogenization of the cell precipitate. From the resulting suspension, a small drop was removed and placed in one extremity of the blade for the performance of smears in duplicate.

5. Observations / analyses:

Type: Determination of the stability of human erythrocytes

Measurements: Duplicate sets of test tubes with physiologic saline solutions (NaCl 0.9%) or aqueous solutions with Roundup® were pre-incubated at 37 °C for 5 min. After the addition of 10 µL of blood samples, homogenization and incubation at 37 °C for 30 min, the flasks were centrifuged for 10 min at 1300 g and the supernatant was analyzed by spectrophotometry at 540 nm. The supernatant and the precipitate were stained with Leishman's stain and analyzed by light microscopy.

Calculations: The dependence of the A_{540} values on the Roundup® concentrations were adjusted by sigmoidal regression lines, given by the Boltzmann equation:

$$A540 = \frac{A1 - A2}{1 + e^{(D - D50)/dD}} + A2$$

where A_1 and A_2 are the A_{540} values that represent the minimum and maximum hemolysis plateaus, D is the Roundup® concentration, D_{50} represents the Roundup® concentration that causes 50% of hemolysis, and dD is the amplitude of the sigmoidal transition between A_1 and A_2.

Type: Micronuclei Frequency in Mice

Observations: The smears were stained with Leishman's stain after drying and the slides were dried at room temperature.

No information on micronucleus evaluation (e.g. number and type of cells evaluated, in- and exclusion criteria, etc.) given.

Statistics: The regression lines were only considered significant when p was lower than 0.05. The comparisons of means between groups were performed by analysis of variance (ANOVA), with $p < 0.05$ indicating statistically significant differences.

KLIMISCH EVALUATION

1. Reliability of study: **Not Reliable.** Determination of the stability of human erythrocytes: Results are not surprising because surfactants are known to compromise cell membrane integrity. Doses not reflective of physiological concentrations of either glyphosate or surfactant.

Micronucleus test in vivo**:** irrelevant route of exposure for surfactant containing formulated products. Results confounded by presence of surfactant toxicity; refer to Heydens (2008)

Comment: Non-guideline, non-GLP studies

Determination of the stability of human erythrocytes

Results attributable to surfactant induced cytotoxicity

Micronucleus test in vivo

Major reporting deficiencies (no information on number of cells evaluated, only graphical documentation of results, no

	information on absolute MN frequencies).
2. Relevance of study:	**Not Relevant** (Test material containing surfactant is not appropriately evaluated in either model).
3. Klimisch code:	**3**

Author(s)	Year	Study title
Vigfusson, N.V. Vyse, E.R.	1980	The effect of the pesticides Dexon, Captan and Roundup on sister chromatid exchanges in human lymphocytes in vitro. Mutation Research Volume: 79 Pages: 53-57

Abstract*

Three pesticides at varying concentration were tested for the induction of SCE [sister chromatid exchanges] in human lymphocytes in vitro. The fungicide, Dexon, sodium (4-(dimethylamino)phenyl)diazene sulfonate, caused the greatest increase in SCE frequency and the response was dose related. The herbicide, Roundup, isopropylamide salt of N-(phosphonomethyl)glycine, had the least effect on SCE requiring the use of much higher concentrations to produce an effect. Limited results were obtained with the fungicide Captan, cis-N-((trichloromethyl)thio)-4-cyclo-hexene-1, 2-dicarboximide, because of toxic levels of the fungicide or solvent used.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item:	Captan; Dexon; Roundup
Active substance(s):	**Captan** - cis-N-((trichloromethyl)thio)-4-cyclo-hexene-1, 2-dicarboximide **Dexon** - sodium (4-(dimethylamino)phenyl)diazene sulfonate; **Roundup** - isopropylamide salt of N-(phosphonomethyl)glycine
Source:	Not reported
Purity:	Not reported

2. Positive control: Ethylmethane sulfonate (EMS)

3. Test material:

Material:	Human lymphocytes
Species:	Human
Sex:	No data
Source:	Two subjects were chosen to act as regular donors of blood cells (not further specified)
Culture medium:	McCoys 5A medium (Gibco) with the addition of 10% fetal calf serum, 1% PHA (Gibco), 1% pen-strep solution (10,000 units penicillin and 10,000 µg streptomycin/mL), and 30.7 µg/mL 5-bromodeoxyuridine (10^{-4} M)

4. Test method:

Study type:	*In vitro* sister chromatid exchange test in human lymphocytes
Guideline:	No

GLP:	No
Guideline deviations:	Not applicable
Duration of treatment:	72h
Dose levels:	Except controls, each 5-mL culture contained: EMS: 1.24, 12.4, 124 mg/mL (10^{-5} , 10^{-4}, 10^{-3} M); Dexon: 2.5, 25.0, 250 mg/mL (10^{-5}, 10^{-4}, 10^{-3} M); Captan: 3.0, 30.0, 300.0 mg/mL (10^{-5}, 10^{-4}, 10^{-3} M); Roundup: 0.25, 2.5, 25.0 mg/mL ($65x10^{-5}$, $65x10^{-4}$, $65x10^{-3}$ M).
Solvents:	Captan dissolved in 7% ethanol followed by a 1:4 dilution in water; All other chemicals were dissolved in distilled water.
No. of replicates:	none

5. Observations/analyses:

Measurements:	For each sample and concentration, 50 well spread and completely differentially stained metaphases were analyzed for SCE frequency from each subject.
Preparation of material:	Stained by the FPG technique
Statistics:	Paired Student's *t*-test were determined for all pairs of data in both subjects

KLIMISCH EVALUATION

1. Reliability of study:	**Not Reliable**
Comment:	Test material was a formulated product containing surfactant. Authors acknowledge cytotoxicity was a confounding factor for data interpretation; since the time of this study, around 1980, surfactant effects on *in vitro* test systems have been well documented. Only very minor changes in SCE were reported, with a limited data set of two donors and a lack of dose-response. Statistical analysis was not feasible with this very limited data set.
2. Relevance of study:	**Not Relevant** (Limited data set, internally consistent findings, no statistics conducted and no dose-response)
3. Klimisch code:	**3**