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 116 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.

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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

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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

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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 binucleated 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 attributable 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

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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

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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

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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

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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

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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) (APVMA, 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.

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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

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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:	*Tradescantia*
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

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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.

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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

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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 phytohemoagglutin (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

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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

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***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**

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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 23% 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

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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)

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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**

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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

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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

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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 $p \leq 0.05$.

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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

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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

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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.

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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 pro-oxidant 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

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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

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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

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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 (ε = 6.22 x 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 (ε = 6.22 x 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 (ε = 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 (ε = 1.56 x 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.

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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**

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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

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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; Garlecron – 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

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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 glyphosate** (Glyphosate not tested; formulation tested)

3. Klimisch code: **3**

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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

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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.

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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 Grünwald 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

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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**

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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

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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 (8.25×10^5 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

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***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

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***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. The 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**

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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

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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.

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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

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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**

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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

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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

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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**

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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

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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.

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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

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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

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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 scenario 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**

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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

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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:
Experiment #1
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

Experiment #2
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:
Experiment #1
10 groups of 10 eggs each (5 eggs per each, two replicas)
Experiment #2
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:

Micronucleus test

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

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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

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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.

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.

Statistics: 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.

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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**

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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

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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:
- healthy
- non-smokers
- non-users of drugs or medications
- 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:
- 26 ± 2 °C
- 12 h light/dark cycle
- *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.

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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:

$$A540 = \frac{A1 - A2}{1 + e^{(D - D_{50})/dD}} + A2$$

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.

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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**

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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

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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

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5. Literature Review of Category 'E' Publications and Other Publications

This section reviews other peer reviewed publications not captured in the previous four subject area reviews, as well as Category 'E' peer reviewed citations, as described in the introduction to this literature review. Publications are presented in OECD Tier II style summaries followed by Klimisch ratings (Klimisch, 1997) then responses/comments on the paper. The first four *in vitro* publications from the same research group are commented on collectively after the fourth summary/Klimisch rating, below.

Author(s)	Year	Study title
Robert Bellé, Ronan Le Bouffant, Julia Morales, Bertrand Cosson, Patrick Cormier et Odile Mulner-Lorillon	2007	L'embryon d'oursin, le point de surveillance de l'ADN endommagé de la division cellulaire et les mécanismes à l'origine de la cancérisation. Journal de la Société de Biologie Volume : 201 Number: 3 Pages: 317-327

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Abstract*

Sea urchin embryo, DNA-damaged cell cycle checkpoint and the mechanisms initiating cancer development (translation from original article)

Cell division is an essential process for heredity, maintenance and evolution of the whole living kingdom. Sea urchin early development represents an excellent experimental model for the analysis of cell cycle checkpoint mechanisms since embryonic cells contain a functional DNA-damage checkpoint and since the whole sea urchin genome is sequenced. The DNA-damaged checkpoint is responsible for an arrest in the cell cycle when DNA is damaged or incorrectly replicated, for activation of the DNA repair mechanism, and for commitment to cell death by apoptosis in the case of failure to repair. New insights in cancer biology lead to two fundamental concepts about the very first origin of cancerogenesis. Cancers result from dysfunction of DNA-damaged checkpoints and cancers appear as a result of normal stem cell (NCS) transformation into a cancer stem cell (CSC). The second aspect suggests a new definition of "cancer", since CSC can be detected well before any clinical evidence. Since early development starts from the zygote, which is a primary stem cell, sea urchin early development allows analysis of the early steps of the cancerization process. Although sea urchins do not develop cancers, the model is alternative and complementary to stem cells which are not easy to isolate, do not divide in a short time and do not divide synchronously. In the field of toxicology and incidence on human health, the sea urchin experimental model allows assessment of cancer risk from single or combined molecules long before any epidemiologic evidence is available. Sea urchin embryos were used to test the worldwide used pesticide Roundup that contains glyphosate as the active herbicide agent; it was shown to activate the DNA-damage checkpoint of the first cell cycle of development. The model therefore allows considerable increase in risk evaluation of new products in the field of cancer and offers a tool for the discovery of molecular markers for early diagnostic in cancer biology. Prevention and early diagnosis are two decisive elements of human cancer therapy.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Roundup
Active substance(s): Glyphosate

Source of test items: Not specified

Lot/Batch #: Not specified

Purity: Not specified (Roundup is presented as a commonly used pesticide/herbicide containing glyphosate (is formulated with surfactants and permeabilisants) as active ingredient.

2. Vehicle and/or positive control: Not specified

3. Test system / cells / organism:

Species: *Not specified (sea urchin embryo used)*

Strain: Not specified

Source: Not specified

Growth conditions: Not specified

4. Test methods:

Assessment of DNA-damage/cell division in sea urchin embryo

Guideline: Non-guideline assays

GLP: Not specified

Guideline deviations: Not applicable

Treatment: Sea urchin embryos were exposed to Roundup at different concentration levels

Dose levels: The evaluated concentration levels were 500 – 2500 fold lower than commonly recommended by the producers were used.

Not further specified

Negative control: Not specified

Positive control: Not specified, Roundup was evaluated together with different pesticide cocktails

Test conditions: Not further specified

Replicates per dose level: No specified

5. Observations/analyses:

Measurements: Not specified

Statistics: Not specified

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KLIMISCH EVALUATION

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

Comment: Documentation insufficient for evaluation.

The publication overview provides information on the general application of the sea urchin embryo model for the prediction of “cancerogenicity”. Only a short reference to another study with a glyphosate-containing herbicide is given. Details of the glyphosate product are not provided. Common surfactants have previously shown the same effects in this model. This model is not appropriate for testing materials containing surfactants because surfactant induced cytotoxicity via membrane

disruption is well documented using *in vitro* systems.

2. Relevance of study: **Not relevant** (Prevention of cell cycle transition was determined for the glyphosate formulation. This model is not appropriate for testing materials containing surfactants.)

3. Klimisch code: **4**

Author(s)	Year	Study title
Marc J., Mulner-Lorillon, O., Durand, G., Belle, R.	2003	Embryonic cell cycle for risk assessment of pesticides at the molecular level. Environmental Chemistry Letters Volume: 1 Number: 1 Pages: 8-12

Abstract*

Cell cycle mechanisms are highly conserved from unicellular eukaryotes to complex metazoans including humans. Abnormalities in the regulation of the cell cycle result in death or diseases such as cancer. Early development of sea urchin has proved to be a powerful model for cell division studies and offers the opportunity to study synchronous cell divisions in the absence of transcriptional control. We have analyzed pesticide induced dysfunctions in the first cell division following fertilization in sea urchin embryos, using Roundup, a widely used pesticide formulation containing isopropylamine glyphosate as the active substance. The pesticide induced cell-cycle dysfunction by preventing the in vivo activation of the universal cell cycle regulator CDK1/cyclin B. We further show that synthesis of the regulator protein, cyclin B, as well as its association to the catalytic protein, CDK1, were not affected by the pesticide. Therefore, our results suggest that the pollutant impedes the processing of the CDK1/cyclin B complex, which is required in its physiological activation. Our studies demonstrate the relevance of sea urchin embryonic cells as a sensitive model to assess pesticide toxicity at the level of the universal cell cycle checkpoints.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Roundup
Active substance(s): Glyphosate isopropylamine salt
Source of test items: Amersham (France)
Lot/Batch #: Not specified
Purity: 170 g/L isopropylamine glyphosate salt

2. Vehicle and/or positive control: Vehicle: Millipore-filtered seawater
Positive control: Not reported

3. Test animals:

Species: Sea urchin (*Sphaerechinus granularis*)
Source: Brest area (France), not further specified
Acclimation period: 5 days
Acclimation environment: Seawater
Environmental conditions: Not reported

4. Test system:

Study type:	Embryonic cell cycle for risk assessment of pesticides at the molecular level
Guideline:	Non-guideline study
GLP:	No
Guideline deviations:	Not applicable
Duration of study:	Treatment for up to 200 min
Dose levels:	0.8 % Roundup (corresponding to 8 mM isopropylamine glyphosate)
Replicates per dose group:	Not reported
Treatment:	Pesticide solutions were adjusted to pH 7.5 and added to the embryo suspension in Millipore filtered seawater. Thousands of embryos were incubated for each experimental determination.
Culturing conditions:	Cultures were performed at 16 °C with constant stirring.

Test methods:

Handling of eggs and embryos

Experiments were performed only on batches exhibiting greater than 90% fertilization and for each experiment, using gametes isolated from a single female. Cultures were observed at short time intervals by Nomarski differential interference contrast (DIC) microscopy for developmental progression.

Preparation of whole embryo extracts and affinity purification of CDK1

Whole extracts from the embryos were prepared by pelleting 200 µL of a 5% egg suspension. The pellets were suspended in electrophoresis sample buffer, vortexed for 15 s and boiled for 3 min. The extracts were clarified by centrifugation.

The CDK1 protein was affinity-purified at different times after fertilization using $p13^{suc1}$-Sepharose beads. Extracts for the measurement of the activation state of CDK1 protein were prepared. For extract preparation for cyclin B protein immunodetection the embryo suspension was rapidly packed by centrifugation. Egg pellets were vortexed in 1 mL ice-cold buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.3, 50 mM sodium fluoride, 10 mM pyrophosphate, 10 mM phenyl-phosphate, 1 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.1 mg/ml soybean trypsin inhibitor, and 0.1% Triton), then immediately frozen in liquid nitrogen and kept at –80 °C. Thawed extracts were homogenized through a 25-gauge needle and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was incubated at 4 °C for 45 min under constant rotation with 25 µL packed $p13^{suc1}$-Sepharose beads. The beads were washed twice in 1 mL ice-cold bead buffer (50 mM Tris-HCl pH 7.4, 5 mM sodium fluoride, 250 mM NaCl, 5 mM ethylene diamine tetraacetic acid (EDTA), 0.1% nonidet-P40 (NP-40), 5 mM ethylene glycol tetraacetic acid (EGTA), 10 µg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 µg/mL soybean trypsin inhibitor, 100 µM benzamidine) and once in 1 mL ice-cold 50 mM Tris-HCl pH 7.5. Beads were suspended in 35 µL electrophoresis sample buffer and boiled for 3 min.

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Analysis of in-vivo CDK1 activation
The assays were performed by incubation of 10 µL beads affinity purified CDK1 protein with 1 µCi [γ-^{32}P]-ATP, 10 µM unlabeled ATP, 2.5 µg histone III-SS in 50 µl buffer containing 60 mM β-glycerophosphate, 30 mM p-nitrophenylphosphate, 25 mM 3-N-morpholino propane sulfonic acid, pH 7.2, 5 mM EGTA, 15 mM MgCl2, 1 mM dithiotreitol, 0.1 mM sodium orthovanadate. After 10 min at 30 °C, the reaction was stopped by chilling the tubes in ice.
Cyclin B Immunodetection
15 µL of the embryo whole extracts or of the affinity-purified CDK1 extracts were resolved by one-dimensional electrophoresis (SDS-polyacrylamide gel). Proteins were transferred to nitrocellulose, stained by Ponceau Red and processed for Western blotting.

5. Observations/analyses:

Cytological observations: Approximately 100 embryos were scored for the developmental stage.

CDK1/Cyclin B activation *in vivo*: The kinase activity of affinity-purified CDK1/cyclin B complex was assayed in standard conditions using histone H1 as a substrate.
Duplicate aliquots of 10 µL were spotted on Whatman P81 phosphocellulose papers, which were washed five times in 1% phosphoric acid and counted in water in a 1450 MicroBetacounter (Wallac, EG & G Instruments).

Analysis of Cyclin B by Immunoblot: Western blots were performed using the anti-cyclin B antibody detected with goat anti-rabbit IgG-AP conjugate (Bio-Rad) using the BCIP-NBT reagent (Fluka). Densitometric analyses of the immunoblots were performed using the domain public NIH image program.

Statistics: Not reported

KLIMISCH EVALUATION

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

Comment: Mechanistic study. Outcome with little additional information compared to the authors' previously published work. Non-standard, non-guideline. Commonly used surfactants have previously shown the same effects in this model.

2. Relevance of study: **Not relevant** (Prevention of cell cycle transition was determined for the glyphosate formulation. This model is not appropriate for testing materials containing surfactants because surfactant induced cytotoxicity via membrane disruption is well documented using *in vitro* systems.)

3. Klimisch code: **3**

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Author(s)	Year	Study title
Marc, J. Mulner-Lorillon, O. Belle, R.	2004	Glyphosate-based pesticides affect cell cycle regulation Biology of the Cell Volume: 96 Pages: 245-249

Abstract*

Cell-cycle dys-regulation is a hallmark of tumor cells and human cancers. Failure in the cell-cycle checkpoints leads to genomic instability and subsequent development of cancers from the initial affected cell. A worldwide used product Roundup 3plus, based on glyphosate as the active herbicide, was suggested to be of human health concern since it induced cell cycle dysfunction as judged from analysis of the first cell division of sea urchin embryos, a recognized model for cell cycle studies. Several glyphosate-based pesticides from different manufacturers were assayed in comparison with Roundup 3plus for their ability to interfere with the cell cycle regulation. All the tested products, Amega, Cargly, Cosmic, and Roundup Biovert induced cell cycle dysfunction. The threshold concentration for induction of cell cycle dysfunction was evaluated for each product and suggests high risk by inhalation for people in the vicinity of the pesticide handling sprayed at 500 to 4000 times higher dose than the cell-cycle adverse concentration.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Amega, Cargly, Cosmic, Roundup Biovert, Roundup 3plus
Active substance(s):	Glyphosate isopropylamine salt
Source:	Amega – CFPI Nufarm; Cargly – Cardel; Cosmic – Calliope; Roundup Biovert and Roundup 3plus – Monsanto.
Lot/Batch #:	Not reported
Purity:	Amega – 360 g/L glyphosate; Cargly – 360 g/L glyphosate; Cosmic – 360 g/L glyphosate; Roundup Biovert – 360 g/L glyphosate; Roundup 3plus – 170 g/L glyphosate.

2. Vehicle and/or positive control: Vehicle: Millipore-filtered seawater
Positive control: Not reported

3. Test animals:

Species:	Sea urchin (*Sphaerechinus granularis*)
Source:	Brest area (France), not further specified
Acclimation period:	5 days
Acclimation environment:	Seawater
Environmental conditions:	Not reported

4. Test system:

Study type:	Cell cycle analysis by means of microscopy

Guideline: Non-guideline study

GLP: No

Guideline deviations: Not applicable

Duration of study: Treatment for up to 240 min

Dose levels: Amega: Control, 0.1 mM, 1 mM;
Cargly: Control, 0.1 mM, 1 mM;
Cosmic: Control, 0.1 mM, 1 mM;
Roundup 3plus: Control, 2 mM, 8 mM;
Roundup Biovert: Control, 1 mM, 10 mM;

Replicates per dose group: 5 - 10 independent experiments.

Treatment: Pesticide solutions were adjusted to pH 7.5 before addition to the embryos suspended in Millipore-filtered seawater. Thousands of embryos were incubated for each experimental determination from which around one hundred were scored for the developmental stage.

Culturing conditions: Embryos were cultured at 16 °C with constant stirring.

Test methods: Handling of eggs and embryos
Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22 µm-Millipore-filtered seawater, rinsed and collected by centrifugation. For fertilization, eggs were suspended in Millipore-filtered sea-water (5 % suspension) containing 0.1 % glycine. Dilute sperm was added to the eggs and withdrawn after fertilization membrane elevation. Experiments were only performed on batches exhibiting greater than 90 % fertilization and each experiment used gametes from a single female.
Sea urchin development and cytological observations
Embryos were observed at short time intervals by phase contrast microscopy for developmental progression. At various times after fertilization, 0.2 mL aliquots of the egg suspension were fixed for at least 2 hours in 0.5 mL methanol/glycerol (3:1) in the presence of the DNA dye bisbenzimide (0.1 µg/mL), mounted in 50 % glycerol and observed under fluorescence microscopy.

5. Observations/analyses:

Measurements: At different times following fertilization, developmental stage and chromatin state were observed by phase contrast microscopy and fluorescence microscopy after DNA staining, respectively.

Statistics: Not reported

KLIMISCH EVALUATION

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

Comment: Non-standard, non-guideline study. Commonly used surfactants have previously shown the same effects in this model.

2. Relevance of study: **Not relevant** (Prevention of cell cycle transition was

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determined for the glyphosate formulation. This model is not appropriate for testing materials containing surfactants because surfactant induced cytotoxicity via membrane disruption is well documented using *in vitro* systems.

3. Klimisch code: **3**

Author(s)	Year	Study title
Marc, J. Belle, R. Morales, J. Cormier, P. Mulner-Lorillon, O.	2004b	Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. Toxicological Sciences Volume: 82 Pages: 436-442

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Abstract*

A glyphosate containing pesticide impedes at 10 mM glyphosate the G2/M transition as judged from analysis of the first cell cycle of sea urchin development. We show that formulated glyphosate prevented dephosphorylation of Tyr 15 of the cell cycle regulator CDK1/cyclin B in vivo, the end point target of the G2/M cell cycle checkpoint. Formulated glyphosate had no direct effect on the dual specific cdc25 phosphatase activity responsible for Tyr 15 dephosphorylation. At a concentration that efficiently impeded the cell cycle, formulated glyphosate inhibited the synthesis of DNA occurring in S phase of the cell cycle. The extent of the inhibition of DNA synthesis by formulated glyphosate was correlated with the effect on the cell cycle. We conclude that formulated glyphosate's effect on the cell cycle is exerted at the level of the DNA-response checkpoint of S phase. The resulting inhibition of CDK1 cyclin B Tyr 15 dephosphorylation leads to prevention of the G2/M transition and cell cycle progression.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Roundup 3plus
Active substance(s): Glyphosate isopropylamine salt
Source: Cluzeau Info Labo (France)
Lot/Batch #: Not reported
Purity: 170 g/L glyphosate isopropylamine salt

2. Vehicle and/or positive control: Vehicle: Millipore-filtered seawater
Positive control: aphidicolin (DNA polymerase I inhibitor)

3. Test animals:

Species: Sea urchin (*Sphaerechinus granularis*)
Source: Brest area (France), not further specified
Acclimation period: 5 days
Acclimation environment: Seawater
Environmental conditions: Not reported

4. Test system:

Study type: Cell cycle analysis: DNA-response checkpoint, cell cycle transition (G2/M)

Guideline: Non-guideline study

GLP: No

Guideline deviations: Not applicable

Duration of study: Treatment for up to 175 min

Dose levels: Control; and
1 % formulated glyphosate i.e. 10 mM equivalent glyphosate (Formulated glyphosate).

Replicates per dose group: 3 - 4 independent experiments.

Treatment: Pesticide solutions were adjusted to pH 7.5 before addition to the embryos suspended in Millipore-filtered seawater. Thousands of embryos were incubated for each experimental determination from which around one hundred were scored for the developmental stage.

Culturing conditions: Embryos were cultured at 16 °C with constant stirring.

Test conditions: Handling of eggs and embryos and cytological observations
Spawning of gametes was induced by intracoelomic injection of 0.1 M acetylcholine. Eggs were collected in 0.22 µm Millipore-filtered seawater and rinsed twice. For DNA synthesis determination experiments, eggs were dejellied by swirling in 3.5 mM citric acid (pH 5) and rinsed in filtered seawater prior to fertilization. For fertilization, eggs were suspended in Millipore-filtered seawater (5% suspension) containing 0.1% glycine. Diluted sperm was added to the eggs and withdrawn after fertilization envelope elevation. Experiments were only performed on batches exhibiting greater than 90% fertilization, and each experiment used gametes from a single female. For some experiments, 0.2 mL aliquots of the embryo suspension at various times after fertilization were fixed in methanol/glycerol (3:1) in the presence of the DNA dye bisbenzimide (0.1 µg/mL) and were mounted in 50% glycerol. Chromatin state observation under fluorescence microscopy was used to determine metaphase stage timing.

Affinity purification of CDK1/cyclin B from embryos
The CDK1 protein was affinity purified at different times after fertilization using $p13^{suc1}$-Sepharose beads. Every 10 min after fertilization, embryos were rapidly packed by centrifugation, immediately frozen in liquid nitrogen, and kept at -80 °C until further processing. Embryos were in ice-cold buffer (60 mM β-glycerophosphate pH 7.2, 15 mM p-nitrophenyl phosphate, 25 mM 4-morpholinepropanesulfonic acid (MOPS), 15 mM ethylene glycol tetraacetic acid (EGTA), 15 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM disodium phenylphosphate, 10 µg/mL soybean trypsin inhibitor (SBTI), 100 µM benzamidine, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.1% Triton). The homogenates were clarified by centrifugation. The CDK1/cyclin B complex was isolated by incubating the supernatant in the presence of $p13^{suc1}$-Sepharose beads. The beads were collected by centrifugation, washed twice in ice-

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cold bead buffer and once in ice-cold 50mMTris-HCl pH 7.5. Proteins were eluted by boiling for 3 min in electrophoresis sample buffer.

Western blot analysis

15 µl of the affinity-purified proteins were resolved by one-dimensional electrophoresis (SDS-polyacrylamide gel). Proteins were transferred to nitrocellulose, stained with Ponceau red and processed for Western blotting.

Phosphatase assays

The specific 3-*O*-methylfluorescein phosphate (OMFP) artificial substrate was used for the estimation of cdc25 activity. Pure human cdc25C was assayed. Embryo extracts were prepared as described for the CDK1/cyclin B purification procedure, except that the homogenization buffer was 80 mM β-glycerophosphate pH 7.3, 20 mM EGTA, 15 mM $MgCl_2$, 1 mM DTT, 25 µg/mL aprotinin, 25 µg/mL leupeptin, 1mM benzamidine, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 10 µg/mL soybean trypsin inhibitor (SBTI), 10 µg/mL pepstatin A. Supernatants used for phosphatase assays were from metaphase-stage embryos as judged by the chromatin state.

Phosphatase assays were performed at 30 °C for at least 1 h. The mixture contained either commercial recombinant cdc25C or embryo extracts in substrate buffer (500 µM 3-*O*-methylfluorescein phosphate (OMFP), 100 mM Tris-HCl pH 8.2, 40 mM NaCl, 1mMDTT, 10mMglutathione, 20% glycerol).

Determination of DNA synthesis in vivo

10 min post-fertilization, methyl-^{3}H-thymidine (10 µCi/ml final concentration), was added to the embryo suspension. Formulated glyphosate or aphidicolin (positive control, 10 µg/mL final) were added 10 min post-fertilization. At different times thereafter, embryos were rapidly packed by centrifugation, rinsed in Millipore-filtered seawater, and suspended in ice-cold 20% trichloroacetic acid (TCA). Aliquots were taken for the determination of total thymidine uptake. After precipitation over night at 4 °C, the pellets were collected by centrifugation, washed with 20% TCA and dissolved in 0.125MNaOH.

5. Observations/analyses:

Cytological observations: Approximately 100 embryos were scored at short time intervals by phase contrast microscopy for cytokinesis.

Chromatin state observation under fluorescence microscopy was used to determine metaphase stage timing.

Western blot analysis: Anti-cyclin B antibody, anti-PSTAIR and anti p-Tyr were used. Membranes were incubated with peroxidase-coupled secondary antibodies (Biorad): goat anti-mouse IgG (H + L)/HRP conjugate for PSTAIR detection and goat anti-rabbit IgG (H + L)/HRP conjugate for cyclin B detection. When needed, membranes were stripped and processed as above with a new antibody. The antigen–antibody complexes were revealed by the chemiluminescence system according to the manufacturer's instructions (Amersham Biosciences).

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Phosphatase assays:	Absorbance was measured at 477 nm in a Pharmacia Biotech Ultrospec 2000 spectrophotometer. Activity of pure cdc25 C was 0.12 OD units per hour.
Determination of DNA synthesis *in vivo*:	Radioactivity was measured on duplicate 500 µL aliquots of the dissolved samples in the presence of Optiphase Supermix scintillation liquid in a 1450 Wallac MicroBeta-Counter. The protein content was measured on the remaining sample using the BCA protein assay kit
Statistics:	Not reported

KLIMISCH EVALUATION

1. Reliability of study:	**Not Reliable**
Comment:	Non-standard, non-guideline study. Commonly used surfactants have previously shown the same effects in this model.
2. Relevance of study:	**Not relevant** (Prevention of cell cycle transition was determined for the glyphosate formulation. This model is not appropriate for testing materials containing surfactants because surfactant induced cytotoxicity via membrane disruption is well documented using *in vitro* systems.
3. Klimisch code:	**3**

Comments based on Monsanto web site response
http://www.monsanto.com/products/Documents/glyphosate-background-materials/Response_ISIS_apr_06.pdf

- Marc and her colleagues conducted *in vitro* studies using sea urchins. They have now published a number of articles based on the faulty premise that Roundup enhances the ability of glyphosate to get into cells to disrupt the cell cycle. While they measure a variety of cellular/molecular endpoints in these studies, the results are not reflective of cellular effects in real-life systems since non-specific changes in cell membrane function have been shown to occur due to surfactants and may also result from other changes in the culture medium such as effects on pH and calcium levels. Note that when the sea urchin embryos are placed back in normal medium they develop into normal sea urchins, indicating a lack of any permanent biological effect.
- When surfactants found in products such as bath gels and shampoos that humans tested in the same sea urchin model (Amouroux, 1999; ref. Doc L Table 3 and included in Doc K) sea urchin assay they produced the same results as Marc *et al.* did ...cell cycle delays.
  - http://www.ncbi.nlm.nih.gov/pubmed/9828259?dopt=Abstract
- Other researchers have found that caffeine also alters cell division in sea urchin embryos
  - http://www.ncbi.nlm.nih.gov/pubmed/9276510?dopt=Abstract
- The sea-urchin test system is not relevant to predicting adverse effects on human health.

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The following two recent publications, by Heu et al. (2012a; 2012b) are commented on collectively after the second summary/Klimisch rating, below .

Author(s)	Year	Study title
Heu, C., Berquand, A., Elie-Caille, C., Nicod, L.	2012a	Glyphosate-induced stiffening of HaCat keratinocytes, a Peak Force Tapping study on living cells. Journal of Structural Biology Volume: 178 Number: 1 Pages: 1-7

Abstract*

The skin is the first physiological barrier, with a complex constitution that provides defensive functions against multiple physical and chemical aggressions. Glyphosate is an extensively used herbicide that has been shown to increase the risk of cancer. Moreover there is increasing evidence suggesting that the mechanical phenotype plays an important role in malignant transformation. Atomic force microscopy (AFM) has emerged within the last decade as a powerful tool for providing a nanometer-scale resolution imaging of biological samples. Peak Force Tapping (PFT) is a newly released AFM-based investigation technique allowing extraction of chemical and mechanical properties from a wide range of samples at a relatively high speed and a high resolution. The present work uses the PFT technology to investigate HaCaT keratinocytes, a human epidermal cell line, and offers an original approach to study chemically-induced changes in the cellular mechanical properties under near-physiological conditions. These experiments indicate glyphosate induces cell membrane stiffening and the appearance of cytoskeleton structures at a subcellular level, for low cytotoxic concentrations whereas cells exposed to IC50 (inhibitory concentration 50%) treatment exhibit control-like mechanical behavior despite obvious membrane damages. Quercetin, a well-known antioxidant, reverses the glyphosate-induced mechanical phenotype.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate
Active substance(s): Glyphosate
Source of test items: Not reported
Lot/Batch #: Not specified
Purity: Not specified

2. Vehicle and/or positive control: Not reported

3. Test system / cells:

Cell lines: Immortalized human HaCaT cell line
Source: ATCC, Teddington, UK
Culture conditions: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; D. Dutscher, Brumath, France) and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 ^{0}C. The keratinocytes were grown to confluence in 75 cm^2 culture flasks. The medium was removed

every 48 h, and cells were subcultured every 7 days at a ratio of 1:6.

4. Test methods:

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Determination of IC50 (Inhibitory concentration 50%: The *in vitro* conditions to study the glyphosate-induced stress were determined by carrying out cytotoxicity assays on 96-well microtiter plate HaCaT cultures with different glyphosate concentrations. The cytotoxic profiles remained unchanged from 6 to 18 h of contact with glyphosate, showing a similar 50% inhibitory concentration (IC50) value around 30 mM. We chose to test 30 mM to observe achieved events and 15 mM (80% cytotoxicity) for ongoing events. These concentrations approximate field spray dilutions of 2500 to 5000 ppm glyphosate), but without pH adjustment for the glyphosate acid.

<u>Atomic Force Microscopy (AFM)</u>

Conditions: HaCaT cells were seeded in 50 mm glass bottom Petri dishes (Willco Wells BV, Amsterdam, The Netherlands) at a density of 5 x 10^5 cells in 3 mL DMEM supplemented with 10% (v/v) FCS and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C for 24 h for cell attachment.

Treatment: Cultured cells were exposed to several concentrations of glyphosate in FCS-free media depending on different incubation periods: 40 and 53 for 0.5 h; 15 and 30 for 6 and 18 h. A 100 mM quercetin treatment was tested concomitantly to 18 h glyphosate conditions.
Following exposure the cells were washed with pre-warmed FCS-free media and imaged at ambient temperature which was found to be 32 °C and over a maximum time of 90 min to be sure that the cells were still healthy and adherent during the imaging.

Replicates per dose level: Not reported

5. Observations/analyses:

Measurements: <u>Tensile modulus calculation and fit model</u>

The YM (Young's modulus) or tensile modulus is obtained by dividing the tensile stress by the tensile strain and is related to the stiffness of the scanned material.
By default, the AFM's software (Nanoscope V8.2) uses a DMT fit to extract the Young's modulus from each force curve. The obtained value is an average of 300,000 force curves.
The data was also processed outside of the AFM software using the Sneddon model (Sneddon, 1965). Although, only one probe was used for the entire series of experiments, the Young's modulus calculated from the exported force curves is an average of 27,000 data points and Young's modulus values extracted using a DMT fit are superimposed to those of another serie of experiments carried out in the same conditions.

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Analysis: AFM imaging was carried out on a Bioscope Catalyst™ (Bruker, Billerica, USA). AFM probes used in Peak Force Quantitative Nanomechanical Mapping (PFQNM) mode were ScanAsyst-Fluid (Bruker, Billerica, USA) probes. The Young's moduli were calculated by using a Sneddon fit (Sneddon, 1965).

KLIMISCH EVALUATION

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

Comment: Non-guideline *in vitro* tests with no control for low pH effects. Minor reporting deficiencies (source and purity of glyphosate, replicates per dose level)

2. Relevance of study: **Not Relevant** (*in vitro* data on the effects on an immortalized epidetmal cell line does consider low exposure potential due to *stratum cornea* protection. Inappropriate test substance if not adjusted for pH: low pH glyphosate acid is not in formulated glyphosate based products)

3. Klimisch code: **3**

Author(s)	Year	Study title
Heu, C., Elie-Caille, C., Mougey, V., Launay, S., Nicod, L.	2012b	A step further towards glyphosate-induced epidermal cell death: Involvement of mitochondrial and oxidative mechanisms. Environmental Toxicology and Pharmacology Volume: 34 Number: 2 Pages: 144-153

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Abstract*

A deregulation of programmed cell death mechanisms in human epidermis leads to skin pathologies. We previously showed that glyphosate, an extensively used herbicide, provoked cytotoxic effects on cultured human keratinocytes, affecting their antioxidant capacities and impairing morphological and functional cell characteristics. The aim of the present study, carried out on the human epidermal cell line HaCaT, was to examine the part of apoptosis plays in the cytotoxic effects of glyphosate and the intracellular mechanisms involved in the apoptotic events. We have conducted different incubation periods to reveal the specific events in glyphosate-induced cell death. We observed an increase in the number of early apoptotic cells at a low cytotoxicity level (15%), and then, a decrease, in favour of late apoptotic and necrotic cell rates for more severe cytotoxicity conditions. At the same time, we showed that the glyphosate-induced mitochondrial membrane potential disruption could be a cause of apoptosis in keratinocyte cultures.

* Quoted from article

MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate
Active substance(s): Glyphosate
Source of test item: Sigma-Aldrich, St. Louis, MO, USA
Lot/Batch #: Not specified
Purity: ≥ 95% (powder)

2. Vehicle and/or positive control: Not reported

3. Test system / cells:

Cell lines: Immortalized human HaCaT cell line (human keratinocytes)
Source: ATCC, Teddington, UK
Culture conditions: Cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; D.Dutscher, Brumath, France) and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C. The keratinocytes were grown to confluence in 75 cm^2 culture flasks. The medium was removed every 48 h, and cells were subcultured every 7 days at a ratio of 1:6.

4. Test methods:

Guideline: Non-guideline assay (for all tests)
GLP: No
Guideline deviations: Not applicable (for all tests)

<u>Cytotoxicity assays</u>

Conditions: HaCaT cells were seeded at a density of 1×10^4 cells per well in 100 µL FCS-supplemented DMEM on 96 multiwell culture plates (Nunc, D. Dutscher) and incubated overnight for adherence. The following day, the medium was removed and cells were incubated in FCS-free medium containing increasing concentrations of glyphosate (for nine incubation times in 37 °C–5% CO_2 atmosphere.
Dose concentrations: 0, 5, 10, 20, 30, 40, 50, 60, and 70 mM glyphosate (approximately 850 – 11,800 ppm glyphosate acid)
Incubation (exposure) times: 0.5, 1, 2, 4, 6, 9, 12, 15 and 18 h
Replicates: 3 per concentration

<u>Flow cytometry</u>

Conditions: For all experiments of the flow cytometry study, HaCaT cells were seeded in 25 cm² culture flasks at a density of 5×10^5 cells/ DMEM supplemented with 10% (v/v) FCS and 80 mg/L of gentamicin in a humidified 5% CO_2 atmosphere at 37 °C for 24 h for cell attachment.

<u>Intracellular ROS (H_2O_2)</u>: 2′-7′-Dichlorodihydrofluorescein diacetate (DCFDA) dye was used to study the intracellular hydrogen peroxide generation in HaCaT cells.
Conditions: After 24 h of cell attachment, the cells were washed twice with 2 mL PBS and DCFDA dye was added at a concentration of 20 µM in 2 mL per flask. The flasks were placed in a humidified 5% CO_2 atmosphere at 37 °C for 30 min.

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Treatment: Following incubation, the dye solution was removed, the cells were washed with 2 mL PBS and were treated with several concentrations of glyphosate in FCS-free media depending on different incubation periods: 30, 40, 53 and 70 mM for 0.5 h ; 15, 20, 30 and 45 mM for 6 and 18 h. To ensure the dye specificity, a positive control is carried out, a treatment with tert-Butyl hydroperoxide (tBHP) 15 µM for 18 h.

Apoptosis

Treatment: After 24 h of cell attachment, cultured cells were exposed to several concentrations of glyphosate in FCS-free media depending on different incubation periods: 30, 40, 53 and 70 mM for 0.5 h; 15, 20, 30 and 45 mM for 6 and 18 h.

Mitochondrial transmembrane potential

Treatment: After 24 h of cell attachment, keratinocytes were treated as for the apoptosis study.

5. Observations/analyses:

Measurements: Cell viability, flow cytometry, intracellular ROS (H_2O_2), apoptosis induction, cell morphology

Cytotoxicity assays: After the exposure periods, the reaction medium was removed, and the residual cell viability was measured in each well using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide procedure. Results were expressed as percentage of controls (100% viability) according to the glyphosate concentrations, and the 50% inhibition concentrations (IC50), i.e. the concentrations of glyphosate killing 50% of keratinocytes, were compared according to the four exposure times. Each experiment was done three times, and each determination was carried out in triplicate.

Flow cytometry: Flow cytometry analyses were performed on a FC500 cytometer (Beckman-Coulter, France).

Analyses were performed on no less than 10 000 cells, using the CXPTM software (Beckman-Coulter).

Intracellular ROS (H_2O_2): Following exposure, and after supernatant recovery, the cells were washed with pre-warmed PBS and were collected by trypsinization; whereafter, the trypsin was removed by centrifugation. Cells were resuspended in PBS before flow cytometry.

Apoptosis: Following exposure, and after supernatants recovery, the cells were washed with pre-warmed PBS and were collected by trypsinization; whereafter, the trypsin was removed by centrifugation. The cells were then stained with the FITC annexin V Apoptosis Detection Kit II (BD biosciences, Franklin Lakes, USA) as recommended in the procedure.
To ensure the dye specificity, a positive control with camptothecin and a negative control after a pretreatment with recombinant annexin V were carried out.

Mitochondrial transmembrane potential: The cells were then stained with the mitochondria staining kit (Sigma CS0390) as recommended by the procedure. To ensure the dye specificity, a negative positive control with

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valinomycin was carried out.

In the data representation, a point represents any cell with its red fluorescence intensity in abscissa and its green fluorescence intensity in ordinate.
The changes in the mitochondrial membrane potential were monitored using JC-1 (5,5V,6,6V-tetrachloro-1,1V,3,3V-tetraethylbenzimidazolcarbocyanine iodide).

Statistics: Not reported

KLIMISCH EVALUATION

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

Comment: Non-guideline *in vitro* test with no control for low pH effects. Minor reporting deficiencies (source and purity of glyphosate, replicates per dose level)

2. Relevance of study: **Not Relevant** (*in vitro* data on the effects on an immortalized epidetmal cell-line does consider low exposure potential due to *stratum cornea* protection. Inappropriate test substance if not adjusted for pH; low pH glyphosate acid is not in formulated glyphosate based products)

3. Klimisch code: **3**

GTF Comments on the two Heu et al. (2012a, 2012b) publications

- Doses evaluated appear to be in the range of spray dilutions of glyphosate formulations.
- Glyphosate technical acid evaluated was not reported to be pH adjusted and therefore does not reflect real world exposures to the more neutral pH formulations, which contain glyphosate salts, not glyphosate acid
- The pH range of test concentrations (850-1150 mg/L) is very acidic, approximately 1.7-2-2 pH units. Keeping in mind the pH scale is logarithmic, these values are substantially lower than those of viable skin and *in vitro* cell cultures.
- Exposure potential to live human epidermal skin cells in the field is likely to be considerably lower than the authors have considered. The epidermis is protected by the *stratum cornea*. Human *in vitro* dermal absorption studies for a range glyphosate formulated products are presented in section 5.9.9, showing very a very low dermal absorption of glyphosate; nearly all of the glyphosate is washed off the skin surface after 24 hour exposures (88% to >99% before *stratum cornea* removal; See Section 5.9.9 Dermal Penetration). Therefore, the studies of Heu et al., while representative of glyphosate spray concentrations, are approximately two or more orders of magnitude higher of those which may result for 8-24 hour dermal exposures.

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Author(s)	Year	Study title
Acquavella, J.F. Alexander, B.H. Mandel, J.S. Gustin, C. Baker, B. Chapman,P. Bleeke, M.	2004	Glyphosate Biomonitoring for Farmers and Their Families: Results from the Farm Family Exposure Study Environmental Health Perspectives Volume: 112 Number: 3 Pages: 321-326

Abstract*

Glyphosate is the active ingredient in Roundup agricultural herbicides and other herbicide formulations that are widely used for agricultural, forestry, and residential weed control. As part of the Farm Family Exposure Study, we evaluated urinary glyphosate concentrations for 48 farmers, their spouses, and their 79 children (4–18 years of age). We evaluated 24-hr composite urine samples for each family member the day before, the day of, and for 3 days after a glyphosate application. Sixty percent of farmers had detectable levels of glyphosate in their urine on the day of application. The geometric mean (GM) concentration was 3 ppb, the maximum value was 233 ppb, and the highest estimated systemic dose was 0.004 mg/kg. Farmers who did not use rubber gloves had higher GM urinary concentrations than did other farmers (10 ppb vs. 2.0 ppb). For spouses, 4% had detectable levels in their urine on the day of application. Their maximum value was 3 ppb. For children, 12% had detectable glyphosate in their urine on the day of application, with a maximum concentration of 29 ppb. All but one of the children with detectable concentrations had helped with the application or was present during herbicide mixing, loading, or application. None of the systemic doses estimated in this study approached the U.S. Environmental Protection Agency reference dose for glyphosate of 2 mg/kg/day. Nonetheless, it is advisable to minimize exposure to pesticides, and this study did identify specific practices that could be modified to reduce the potential for exposure.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

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

2. Vehicle and/or positive control: Not applicable

3. Test group:

Number of test persons: 48 farmers, 48 spouses, 79 children

Age: ≥ 4 years (children); 11.5 yr (mean)
Farmers: 45 yr (mean)
Spouses: 42.2 yr (mean)

Sex: Males and females

Inclusion criteria:
- Live on the farm;
- farmers and spouses that have at least 1 child (4-18 yr)
- Farm at least 10 acres within 1 mile of the family residence, to which they planned to apply one or a combination of the

pesticides included in the study: glyphosate, 2,4-D (2,4-dichlorophenoxyacetic acid), or chlorpyrifos. There were no restrictions on using these pesticides before or immediately after the planned on-study application.
- Family members had to be willing to collect all urine voids for five consecutive days: the day before, the day of, and 3 days after the planned pesticide application.
- Farmer and spouse had to be willing to fill out pre- and post-study questionnaires, thereby detailing family activities for the week before the study and the week of the study, and agree to have their on-study pesticide application observed by trained field staff.

4. Test system:

Type: Glyphosate Biomonitoring Study

Collection of data:
- Questionnaire + interview (incl. assessment of demographic and lifestyle factors)
- Field observation of application or related activities, application equipment, meteorological conditions, work practice (incl. protective measures), and family activity patterns
- Urine analyses

Guideline: Non-guideline study

GLP / GCP: Yes

Exposure conditions: No information on application rates reported. Numbers of mixing/loading operations and areas treated are provided.

Sample collection: During the study period, defined as 24 hr before the start of on-study pesticide-related activities (day –1) and continuing for 4 consecutive 24-hr periods (days 0, 1, 2, and 3, respectively), participants collected individual urine voids in 500-mL high-density polyethylene wide-mouth containers.

Field research staff collected the samples daily, monitored compliance, logged each sample in a computer database, and created 24-h composite urine samples with amounts proportional to the volume of each individual urine sample. Samples were shipped frozen to analytical laboratory.

5. Observations / analyses:

Measurements: Urine samples were analyzed for glyphosate concentration using a previously published method (Cowell et al. 1986) modified for urine (FFES 2003). The method employs chelation ion exchange for the concentration and isolation of glyphosate, followed by quantitation using high-performance liquid chromatography with postcolumn reaction and fluorescence detection. The method has a limit of detection (LOD) of 1 µg/L (or 1 ppb) for a 100-mL urine sample. Results were corrected for laboratory analytical recovery and storage stability as determined by analysis of fortified filed samples prepared throughout the study.

Calculations: The systemic dose (= absorbed dose in mg/kg bw) was estimated for all farmers, spouses and children who had detectable urinary levels of glyphosate, by calculating the amount of glyphosate excreted during the study period, adjusting for incomplete excretion and pharmacokinetic

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recovery, and dividing the total corrected excretion by each individual´s body weight.

Statistics: Geometric mean (GM) urinary concentrations were calculated for farmer-applicators as the antilog of the average of the natural log (ln)–transformed urinary concentrations (SAS, version 8.2 for Windows). The standard deviation (SD) was calculated as the antilog of the SD of the ln-transformed urinary concentrations. In these calculations, a value of 0.5 ppb (LOD/2) was assigned for concentrations that were below the LOD. GM concentrations for spouses and children were not calculated because too few of these subjects had detectable concentrations in their urine. *t*-tests and one-way analysis of variance were used to compare GMs for farmers who followed different application practices (SAS, version 8.2). Two-tailed significance tests were used, consistent with the null hypothesis of no relationship between application practices and urinary pesticide concentration.

Glyphosate that was recovered in urine was devided with complete pharmacokinetic recovery by each individual's body weight to yield a systemic dose in milligrams per kilogram body weight.

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KLIMISCH EVALUATION

1. Reliability of study: **Reliable without restrictions**

Comment: Well documented GLP study, which meets requirements of exposure test guidelines. Study design is scientifically acceptable.

2. Relevance of study: **Relevant** (Provides information on real-life exposure to glyphosate in farmers and their families)

3. Klimisch code: **1**

GTF Comments

- The Acquavella et al. (2004) Farm Family Exposure Study (FFES) is depicted by Earth Open Source (EOS) as not reflective of real world exposures and suggest a long term exposure assessment study would be more appropriate.
- EOS references a letter to the Editor by Mage, stating the obvious; increased spatial and temporal monitoring offer greater insight than shorter duration exposure and monitoring studies.
- EOS suggests an alternative exposure monitoring study by Curwin (2007), which reports geometric mean systemic (maximum likely dose to farmers' children (0.11 ug/kg/day, or 0.00011 mg/kg/day) and non-farm children (0.13 ug/kg/day, or 0.00013 mg/kg/day). These systemic doses are comparable to the conservative FFES geometric mean systemic dose to farmers (0.0001 mg/kg/day). However, the FFES provides a great deal of detail on urinary concentrations for farmers with different pesticide handling practices or procedures.
- Curwin (2007) which reports on children biomonitoring data for farm children and non-farm children, taken once "a few days after" application and again one month later. Glyphosate systemic exposures were similar for farm and non-farm children, and all were well below the chronic reference dose or acceptable daily intake.

- The GLP compliant Acquavella et al. (2004) is one of few comprehensive published operator and family exposure monitoring studies for glyphosate available, with time-course measurements for glyphosate; the day before, the day of, and for 3 days after application, reporting human systemic exposures for 48 farmers, their spouses and 79 children.

Below is a summary of the Letter to the Editor by Mage.

Author(s)	Year	Study title
Mage, D.T.	2006	Suggested Corrections to the Farm Family Exposure Study Environmental Health Perspective Volume: 114 Number: 11 Page: A 633

Abstract

No abstract.

[The author of letter claims that the study conducted by Acquavella et al. contains methodological deficiencies. Thus, the author recommends that Acquavella et al. (2004) consider revising their analysis by correcting properly for incomplete urine collection, correcting for the initial condition of prior glyphosate exposure, and a adjusting for the experience of the applicator (lifetime number of application days) as an explanatory variable].

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® Ultra
Active substance(s):	Glyphosate
Description:	Not reported
Source of test medium:	Not reported
Lot/Batch #:	Not reported
Concentration:	Not reported

2. Study addressed: Acquavella et al.(Environmental Health Perspectives (2004), 112, 321-326)

Glyphosate Biomonitoring Study:
- Questionnaire + interview (incl. assessment of demographic and lifestyle factors)
- Field observation of application or related activities, application equipment, meteorological conditions, work practice (incl. protective measures), and family activity patterns
- Urine analyses

KLIMISCH EVALUATION

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

Comment: In this publication the article by Aquavella et al. (Environmental Health Perspective (2004), 114, A 633) is discussed in detail. The author of letter claims that the study conducted by Acquavella et al. contains methodological deficiencies regarding urine collection, initial condition of prior glyphosate exposure, and statistical analysis.

2. Relevance of study: **Relevant** (No original publication but letter to the editor regarding the article by Acquavella et al., 2004)

3. Klimisch code: **Not applicable**

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Author(s)	Year	Study title
Anadon, A. Martinez-Larranaga, M.R. Martinez, M.A. Castellano, V.J. Martinez, M. Martin, M.T. Nozal, M.J. Bernal, J.L.	2009	Toxicokinetics of glyphosate and its metabolite aminomethyl phosphonic acid in rats Toxicology Letters Volume: 190 Pages: 91-95

Abstract*

The toxicokinetics of glyphosate after single 100 $mgkg^{-1}$ intravenous (i.v.) and 400 $mgkg^{-1}$ oral doses were studied in rats. Serial blood samples were obtained after i.v. and oral administration. Plasma concentrations of glyphosate and its metabolite amiomethyl phosphonic acid (AMPA) were determined by HPLC method. After i.v. and oral administration, plasma concentration-time curves were best described by a two-compartment open model. For glyphosate, the elimination half-lives ($T_{1/2\beta}$) from plasma were 9.99 h after i.v. and 14.38 h after oral administration. The total plasma clearance was not influenced by dose concentration or route and reached a value of 0.995 l h^{-1} kg^{-1}. After i.v. administration, the apparent volume of distribution in the second compartment (V_2) and volume of distribution at steady state (V_{ss}) were 2.39 and 2.99 l kg^{-1}, respectively, suggesting a considerable diffusion of the herbicide into tissues. After oral administration, glyphosate was partially and slowly absorbed with a T_{max} of 5.16 h. The oral bioavailability of glyphosate was found to be 23.21%. Glyphosate was converted to AMPA. The metabolite AMPA represented 6.49% of the parent drug plasma concentrations. The maximum plasma concentrations of glyphosate and AMPA were 4.62 and 0.416 μg ml^{-1}, respectively. The maximum plasma concentration of AMPA was achieved at 2.42 h. For AMPA, the elimination half-life ($T_{1/2\beta}$) was 15.08 h after oral administration of glyphosate parent compound.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate
CAS No: 107-83-6
Purity: 95% (w/w)
Source: SIGMA CHEMICAL CO., St Louis, MO, USA

2. Vehicle: Corn oil for oral and glycerol formal for i.v. applications

3. Test animals:

Species: Rat
Strain: Wistar
Source: Charles River Inc., Margate, Kent, UK
Age of test animals at study initiation: Adult
Sex: Male
Body weight: 200-210 g
Acclimation period: Not reported

Diet/Food:	A04 rodent diet, Panlab SL, *ad libitum*, except for 12 hours before dosing
Water:	Water, *ad libitum*
Housing::	Individually housed in polycarbonate cages with sawdust bedding
Environmental conditions:	Temperature: 22 ± 2°C Humidity: 50 ± 10% Air changes: Not reported 12-hour light/dark cycle

4. Test system:

Study type:	Toxicokinetic study
Guideline:	No
GLP:	No
Guideline deviations:	Not applicable
Animals per dose group	The rats were divided into two groups of 80 animals each, one group (Group 1) received glyphosate orally and the other group (Group 2) intravenously.
Group 1	
Dose level:	400 mg/kg bw
Administration:	Rats received a single oral administration of 400 mg/ kg bw gavage in a volume of 0.5 mL corn oil/rat
Group 2	
Dose level:	100 mg/kg bw
Administration:	Rats received a single i.v. injection of 100 mg/ kg bw (in 0.1ml glycerol formal/rat) into the lateral tail vein
Sacrifice:	8 rats per observation time point were killed by cervical dislocation and then exsanguinated.
Time points:	0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h after oral and i.v. administration of glyphosate
Blood sampling and processing:	Blood was withdrawn at each time point and collected in heparinised tubes. Plasma was separated by centrifugation and stored frozen at -80°C until analysis.

5. Observations/analyses:

Clinical signs:	Assessed, but no details provided
Measurement:	Glyphosate and its metabolite AMPA concentrations in plasma were measured by HPLC.
Calculations and statistics:	The mean plasma concentration versus time data were sequentially fitted to 1-, 2- and multiple-compartment models. The 2-compartment model was used to establish toxicokinetic characteristics. Plasma curves of glyphosate after a single i.v. and oral administration and those of AMPA (the main metabolite in plasma) after a single oral administration of glyphosate were fitted to the exponential equations: $C = A_1 e^{-\alpha t} + A_2 e_{-\beta t}$ (i.v.)

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$C = A_1 e^{-\alpha t} + A_2 e_{-\beta t} - A3\ e^{-\kappa a t}$ (p.o.)

The following parameters were also calculated:

- area under the concentration-time curve (AUC)
- total plasma clearance
- mean residence time (MRT) (only for i.v. administration)
- volume of distribution in the central compartment (V_1)
- apparent volume of distribution in the second compartment (V_2)
- volume of distribution at steady state (V_{ss}) (only for i.v. administration)
- maximum drug plasma concentration (C_{max}) after oral administration and the time at which C_{max} was achieved (T_{max}) was determined directly from the concentration versus time curve.

KLIMISCH EVALUATION

1. Reliability of study: **Reliable with restrictions**

Comment: Study report meets basic scientific principles, and is comparable to actual kinetics guidelines (large number of test animals used; standard deviations and mass balance not reported)

2. Relevance of study: **Relevant** (additional information on blood plasma concentrations of glyphosate and AMPA, as well as elimination after oral and i.v. application of glyphosate.)

3. Klimisch code: **2**

GTF Comments

- EOS made one comment on this kinetics study

  "Because glyphosate and AMPA were cleared from blood more slowly after oral dosing, they could be distributed to body tissues to exert systemic toxic effects."
- Intravenous injection is more rapidly excreted than oral simply because of lag time for GI tract absorption and entry into the blood stream. That reported, half-lives for i.v (0.345 and 9.99 hours for α and β phases respectively) versus oral (4.17 and 14.38 hours for α and β phases respectively) are all very rapid elimination kinetics, emphasizing the low duration of any systemic exposure to glyphosate, irrespective of the route.

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Author(s)	Year	Study title
Axelrad, J.C. Howard, C.V. McLean, W.G.	2003	The effects of acute pesticide exposure on neuroblastoma cells chronically exposed to diazinon Toxicology Volume: 185 Pages: 67-78

Abstract*

Speculation about potential neurotoxicity due to chronic exposure to low doses of organophosphate (OP) pesticides is not yet supported by experimental evidence. The objective of this work was to use a cell culture model of chronic OP exposure to determine if such exposure can alter the sensitivity of nerve cells to subsequent acute exposure to OPs or other compounds. NB2a neuroblastoma cells were grown in the presence of 25 µM diazinon for 8 weeks. The OP was then withdrawn and the cells were induced to differentiate in the presence of various other pesticides or herbicides, including OPs and OP-containing formulations. The resulting outgrowth of neurite-like structures was measured by light microscopy and quantitative image analysis and the IC50 for each OP or formulation was calculated. The IC50 values in diazinon-pre-exposed cells were compared with the equivalent values in cells not pre-exposed to diazinon. The IC50 for inhibition of neurite outgrowth by acute application of diazinon, pyrethrum, glyphosate or a commercial formulation of glyphosate was decreased by between 20 and 90% after pre-treatment with diazinon. In contrast, the IC50 for pirimiphos methyl was unaffected and those for phosmet or chlorpyrifos were increased by between 1.5- and 3-fold. Treatment of cells with chlorpyrifos or with a second glyphosate-containing formulation led to the formation of abnormal neurite-like structures in diazinon-pre-exposed cells. The data support the view that chronic exposure to an OP may reduce the threshold for toxicity of some, but by no means all, environmental agents.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test items: **Diazinon**, (o,o-diethyl o-(2-isopropyl-6-methylpyrimidin-4-yl) phosphothioate);
phosmet, (o,o-dimethyl S-phthalimidomethyl phosphorodimethioate);
pirimiphos methyl, (o-(2-diethylamino- 6-methylpyrimidin-4-yl) o,o-dimethyl phosphorothioate);
chlorpyrifos, (o,o-diethyl o-(3,5,6- trichloro-2-pyridyl) phosphorothioate);
glyphosate, (N-(phosphonomethyl) glycine)

Roundup Weed Killer, containing 0.72% (w/v) glyphosate acid as a 9.7 g/L IPA salt of glyphosate in a soluble concentrate;
Tough Weed Killer, containing 5% (w/v) glyphosate trimesium

Sources: Purified pesticides – Chemserve/Greyhound Chromatography, Birkenhead, UK.

Herbicide formulations – Roundup Weed Killer from Solaris, Garden Division of Monsanto, High Wycombe, UK, (Lot No. C8K2767);

Tough Weed Killer from Miracle Gardencare, Godalming, UK, (coded Dec97294)

Purity: ≥ 99% for all pesticides except the formulations

2. Vehicle and/or positive control: Methanol (0.25 % w/v) or dimethyl sulphoxide (DMSO) or serum-containing medium (for non-induction of differentiation)

3. Test system/cells:

(in the following information is only provided for test with glyphosate or glyhosate-containing herbicides)

Cells: Mouse NB2a neuroblastoma, cell line: 89121404

Source: ECACC

Culture medium: High glucose Dulbecco's Modified Eagle's Medium (DMEM) containing Glutamax- 1, and supplemented with 5% (v/v) horse serum, 5% (v/v) foetal calf serum, 100 U/ml penicillin plus 100 µg/ml streptomycin and 25 µg/ml gentamicin

Culture conditions: Temperature: 37°C
Atmosphere: 5% CO_2; humidified

4. Test method:

Study type: Acute pesticide exposure on neuroblastoma cells after chronic exposure of organophosphate

Guideline: None

GLP: No

Guideline deviations: Not applicable

Pre-exposure to diazinon: Diazinon-pre-exposed cells were maintained for either 24 h or 8 weeks before use in maintenance medium containing 25 µM diazinon;
Control cells were maintained under exactly the same condition, but with addition of only the solvent (methanol at 0.25 %) in which the diazinon was dissolved

Exposure to test substances: After the pre-exposure period, cells were added to wells of 48-well culture plates at a density of 9000 cells per cm^2 in normal diazinon-free culture medium.
After 24 h the medium was removed and replaced with serum-free medium containing 1 mM dibutyryl cyclic AMP (without diazinon) to induce differentiation. (Differentation led to the growth of neurite-like structures). At the same time, the test compouns to be tested were added to the medium at a range of concentrations. Control cells were exposed to the vehicle of dilution only (either DMSO or methanol), and negative control cells continued to be grown in serum-containing medium, i.e. there were not induced to differentiate.

Exposure duration: 24 h

Dose levels: For pre-exposure (8 weeks or 24 h): 25 µM diazinon
For acute exposure:
50 – 1000 µM glyphosate
not specified for glyphosate-containing formulations
After exposure cells were fixed and stained with Comassie Blue.

Replicates per combination of substances: 4

5. Observations/analyses:

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Measurements: ≥ 200 cells from six to 20 different fields were evaluated for each experiment microscopically.
An automated image analysis program performed a segmentation and skeletalisation of the images of the cells by serial erosion to single pixel width.
The average length of neuritis per cell for each different treatment was recorded. Toxixicty was measured in terms of a reduction in neurite length of treated differentiating cells compared with untreated differentiating controls, after subtraction of a baseline level on no neurite outgrowth as measured in the cells grown in serum-containing medium.
IC_{50} (the level at which 50% reduction in neurite outgrowth was achieved) was determined in separate experiments from the relationship between neurite outgrowth and the test substance concentration.
Effects of individual pesticides pre-exposed to diazinon were compared to non-pre-exposed cells.

Statistics. The significance of any difference was compared by Students' t -test of IC_{50} values, or of the neurite inhibition at single concentration points.

KLIMISCH EVALUATION

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

Comment: Incorrect chacacterization of glyphosate as an organophosphate pesticide. Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactant are well known for in vitro test systems. Exposure route not relevant for human risk assessment. Rationale for chosen test substance concentration not given.

2. Relevance of study: **Not relevant** (*in vitro* data, do not reflect real *in vivo* exposure situations. Pre-exposure to diazinon is not relevant for this submission,)

3. Klimisch code: **3**

GTF Comment

- Inappropriate test system to evaluate test materials containing membrane damaging surfactants.
- See earlei discussion under *In Vitro* Glyphosate DART/ED Publications under this dossier point (literature review).

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Author(s)	Year	Study title
Benedetti, A. L. Vituri, C.D. Trentin, A.G. Domingues, M.A.C. Alvarez-Silva, M.	2004	The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb® Toxicology Letters Volume: 153 Pages: 227-232

Abstract*

The object of this study was to analyze the hepatic effects of the herbicide Glyphosate-Biocarbo (as commercialized in Brazil) in Wistar rats. Animals were treated orally with water or 4.87, 48.7, or 487 mg/kg of glyphosate each 2 days, during 75 days. Sub-chronic treatment of animals starting from the lowest dose of glyphosate induced the leakage of hepatic intracellular enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), suggesting irreversible damage in hepatocytes. We observed the increase of Kupffer cells in hepatic sinusoid of glyphosate-treated animals. This was followed by large deposition of reticulin fibers, composed mainly of collagen type III. We may conclude that Glyphosate-Biocarbo may induce hepatic histological changes as well as AST and ALT leaking from liver to serum in experimental models.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item: Glyphosate-Biocarb®
Active substance(s): Glyphosate
Source: Sao Jose dos Pinhais, Brazil
Purity: 360 g/L

Surfactant: Polyoxyethyleneamine
Purity: 18% (w/v)

2. Vehicle:
Distilled water

3. Test animals:

Species: Rat
Strain: Wistar
Source: Departamento de Analises Clinicas – CSS – Universidade Federal de Santa Catarina, Florianopolis, SC, Brazil
Age of test animals at study initiation: 90 days
Sex: Male
Body weight: 280-310 g
Acclimation period: Not reported
Diet/Food: Laboratory rat chow, *ad libitum*
Water: Water, *ad libitum*
Housing:: Polyethylene (65 cm × 25 cm × 15 cm) home cages, with sawdust-covered floors. Number of rats per cage not reported.

Environmental conditions: Temperature: 22 ± 2°C
Humidity: Not reported
Air changes: Not reported
12-hour light/dark cycle

4. Test system:

Study type: Sub-chronic study

Guideline: No

GLP: No

Guideline deviations: Not applicable

Duration of study: 75 days

Dose levels: 0 (distilled water), 4.87, 48.7, or 487 mg/kg of Glyphosate-Biocarb® diluted in water

Animals per dose group: Control group: n=16
Experimental groups: n=14 each group

Administration route: Gavage

Administration volume: 0.5 ml/kg

Administration frequency: Each 2 days

Blood sampling and processing: Blood samples collected from all animals by percutaneous cardiac puncture before sacrifice.
Blood samples were transferred to test tubes and allowed to stand for 30 min to clot before being centrifuged at $300 \times g$ for 10 min. Sera were obtained by centrifugation and stored at 4 C.

Tissue sampling and processing: Liver samples were obtained from all animals by surgical processing.
Sections of 2 µm were mounted in glass slides covered with saline.

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5. Observations/analyses:

Measurements of liver enzymes

Mortality: Not reported

Clinical signs: Not reported

Body weight: Not reported

Food- and water consumptions: Not reported

Haematology: Not reported

Clinical chemistry: AST and ALT levels

Urine analysis Not reported

Sacrifice/pathology: Not reported

Organ weights: Not reported

Histopathology: Liver tissue samples were evaluated after haematoxylin–eosin staining.
The liver samples were also assessed for the deposition of reticulin fibers by Gomory trichromic staining.

Statistics: Parametric data, expressed as mean ± S.E.M., were analyzed by one-way variance Newman–Keuls for ALT and AST tests. Differences were considered to be statistically significant when

$p < 0.05$

KLIMISCH EVALUATION

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

Comments: Study report meets basic scientific principles. Study design and documentation is insufficient for assessment.

2. Relevance of study: **Not relevant** (Study design not sufficient for assessment. Toxicity attributable to high oral dosing of surfactant component. There are several reporting deficiencies.)

3. Klimisch code: **3**

GTF Comment

- Effects noted at the high dose of 487 mg/kg/day formulated product in this study are equivalent to approximately 87 mg/kg/day POEA surfactant.
- High dose surfactant effects are noted at doses well below those reported in the US EPA Alky Amine Polyalkoxylates Human Health Risk Assessment (http://www.regulations.gov/search/Regs/home.html#documentDetail?R=09000064809b983b), in which a surfactant within the same class as POEA demonstrated a range of adverse effects, including mortality, at 30 mg/kg/day and a NOAEL of 15 mg/kg/day.
- Overt toxicity reported in Benedetti et al (2004) is consistent with the ingestion of an irritating surfactant and not glyphosate.

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Author(s)	Year	Study title
Mesnage, R. Clair, E. Gress, S. Then, C. Szekacs, A. Seralini, G.E.	2012b	Cytotoxicity on human cells of Cry1Ab and Cry 1Ac *Bt* insecticidal toxins alone or with a glyphosate-based herbicide. Journal of Applied Toxicology doi: 10.1002/jat.2712. [Epub ahead of print]

Abstract*

The study of combined effects of pesticides represents a challenge for toxicology. In the case of the new growing generation of genetically modified (GM) plants with stacked traits, glyphosate-based herbicides (like Roundup) residues are present in the Roundup-tolerant edible plants (especially corns) and mixed with modified *Bt* insecticidal toxins that are produced by the GM plants themselves. The potential side effects of these combined pesticides on human cells are investigated in this work. Here we have tested for the very first time Cry1Ab and Cry1Ac *Bt* toxins (10 ppb to 100 ppm) on the human embryonic kidney cell line 293, as well as their combined actions with Roundup, within 24 h, on three biomarkers of cell death: measurements of mitochondrial succinate dehydrogenase, adenylate kinase release by membrane alterations and caspase 3/7 inductions. Cry1Ab caused cell death from 100 ppm. For Cry1Ac, under such conditions, no effects were detected. The Roundup tested alone from 1 to 20 000ppm is necrotic and apoptotic from 50ppm, far below agricultural dilutions (50% lethal concentration 57.5ppm). The only measured significant combined effect was that Cry1Ab and Cry1Ac reduced caspases 3/7 activations induced by Roundup; this could delay the activation of apoptosis. There was the same tendency for the other markers. In these results, we argue that modified *Bt* toxins are not inert on non-target human cells, and that they can present combined side-effects with other residues of pesticides specific to GM plants.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® GT Plus Cry1Ab toxin Cry1Ac toxin
Active substance(s):	Glyphosate Cry1Ab toxin Cry1Ac toxin
Source of test items:	Roundup® GT Plus: Monsanto, Anvers, Belgium Cry1Ab toxin and Cry1Ac toxin: unspecified laboratories (described elsewhere)
Lot/Batch #:	Not specified
Purity:	Roundup® GT Plus: 450 g/L glyphosate acid (N-phosphonomethyl-glycine) Cry1Ab toxin and Cry1Ac toxin: unspecified
Homologation:	Roundup® GT Plus: 2020448 Cry1Ab toxin and Cry1Ac toxin: unspecified

2. Vehicle and/or positive control:

Not reported

3. Test system / cells:

Cell lines: Human embryonic kidney (HEK) 293 cell line (ECACC 85120602)

Source: Sigma-Aldrich (Saint-Quentin Fallavier, France)

Culture conditions: Cells were grown in phenol red-free EMEM containing 2 mM glutamine, 1% nonessential amino acid, 100 U/mL of antibiotics (a mixture of penicillin, streptomycin and fungizone), 10 mg/mL of liquid kanamycin and 10% fetal bovine serum.

Cells were grown at 37 °C (5% CO_2, 95% air) during 24 h to 80% confluence.

4. Test methods:

Mitochondrial respiration assay (MTT): Assessment of cell viability

ToxiLight® assay: Bioluminescent assay for quantitative measurement of cell membrane damage

Caspase-Glo® 3/7 assay: Assessment of caspase activity or apoptosis induction

Guideline: Non-guideline assays

GLP: No

Guideline deviations: Not applicable

Cell treatments for all tests: Cells at 80% confluence in 48- or 96-well plates were washed with serum-free EMEM, in order to avoid other combined effects, and then exposed to various concentrations of *Bt* toxins or Roundup GT Plus in EMEM serum-free medium for 24 h.

Dose levels: *Bt* toxins: 10 ppb - 100 ppm (in the range of GM plant production).
Roundup: 1 - 20 000 ppm (the latter is the agricultural dilution).
Combined effects: measured by mixing LC_{50} of Roundup with *Bt* toxin: 1, 10, 100 ppm.

Test methods: For the methods used authors refer to: Benachour N, Séralini GE. 2009. Glyphosate formulations induce apoptosis and necrosis in human umbilical, embryonic, and placental cells. *Chem. Res. Toxicol.* **22**: 97–105.

Replicates per dose level: The experiments were repeated at least 3 times in different weeks on 3 independent cultures (n = 9).

5. Observations/analyses:

Measurements: Cytotoxicity - mitochondrial respiration level, by succinate dehydrogenase (SD) activity assessment;
Membrane damage - determination of adenylate kinase (AK) activity;
Apoptosis induction – assessment of caspase 3 and 7 activities.

Mitochondrial respiration assay (MTT): Analysis: Succinate dehydrogenase activity measurement. The optical density was measured at 570 nm using a Mithras LB 940 luminometer.

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ToxiLight® assay	Analysis: Bioluminescent assay, membrane degradation measured by the intracellular AK release in the medium (necrosis marker). Luminescence measured using a Mithras LB 940 luminometer.
Caspase-Glo® 3/7 assay	Analysis: Luminescence measured using a Mithras LB 940 luminometer.
Statistics:	LC_{50} values were calculated by a nonlinear regression using a sigmoid (five-parameter) equation with the GraphPad Prism 5 software. All data were presented as the means ± standard errors (SEs). Statistical differences were determined by Student t-test using significance levels at $P<0.01$ and $P<0.05$.

KLIMISCH EVALUATION

1. Reliability of study:	**Not reliable**
Comment:	Non-guideline, non-GLP *in vitro* tests meeting scientific principles. Deficiencies: No positive controls were specified, test conditions not described (referenced to a description elsewhere). Exceedingly high doses and an inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems.
2. Relevance of study:	Relevant with restrictions (Due to reliability. The assessed combinatory effects are of limited relevance)
3. Klimisch code:	**3**

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Monsanto Comments: Mesnage et al. (2012).

General Statement:

This publication presents no new findings relevant to a safety assessment of glyphosate-based herbicides or Cry proteins Cry1Ab or Cry1Ac. The experimental system placed high concentrations of herbicide and Cry proteins directly on living cells in culture, which is not viewed by public health experts as relevant methodology for evaluating risks to humans.

Animal data and human experience contradicts findings of Petri dish experiments with glyphosate-surfactant herbicides. Surfactants are routinely added to herbicide formulations to break down the waxy coating on plants and allow for efficient absorption. Surfactants also occur in soaps, shampoos, dishwashing detergents, and laundry products, which account for nearly all (over 99%) of consumer surfactant exposure.

Numerous studies have been conducted where animals were administered very high dosages of Cry proteins and suffered no ill effects. High-dose animal toxicity testing using Cry1Ab and Cry1Ac demonstrates no toxic effects at doses thousands of times higher than any potential human intake. Moreover, Cry proteins are destroyed by heating and digested in the gastro-intestinal tract. As a result, intact Cry protein is not detectible in the serum and tissues of animals that have ingested feed containing Cry proteins. As with glyphosate, the safety of Cry1Ab and Cry1Ac has been reviewed and confirmed by regulatory agencies around the globe.

Comments:

1) **Glyphosate has an excellent human health and environmental profile and a long history of safe use in more than 130 countries.** This has been a key factor in the acceptance of glyphosate products as among the most widely used herbicides in the world. When used according to label directions, these products present no unreasonable risk of adverse effects to human health or the environment. This is confirmed by the extensive studies as well by the first-hand experience of millions of farmers and home gardeners who have used this product.

   Glyphosate, the active ingredient in Roundup branded agricultural products and other glyphosate based herbicide formulations, inhibits an enzyme that is essential to plant growth; this enzyme is not found in humans or other animals, which explains the generally low acute toxicity of glyphosate in humans and animals (Franz et al., 1997). Comprehensive toxicological studies in animals have demonstrated that glyphosate does not cause cancer, birth defects, mutagenic effects, nervous system effects or reproductive problems (U.S. EPA, 1993; Williams et al., 2000; Williams et al., 2012); European Commission, 2002; JMPR/WHO, 2004; Mink et al., 2011). In fact, after a thorough review of all toxicology data available, the U.S. EPA concluded that glyphosate should be classified in Category E ("Evidence of Non-carcinogenicity in Humans"), the most favorable category possible (U.S. EPA, 1993).

2) **Artificial conditions.** Direct exposure to cells in culture bypasses normal processes limiting absorption and cellular exposure and avoids normal metabolism, excretion, serum protein binding, and other factors that would protect cells in the intact organism.

3) **Glyphosate-surfactant levels tested are not relevant to real exposures.** Anadon et al. (2009) dosed rates with 400 mg/kg of glyphosate, a massive dose relative to any environmental exposure, and achieved peak modeled plasma concentrations of glyphosate of approximately 5 ug/mL (5mg/L or 5 ppm). Assuming linear kinetics, the maximum allowable US daily intake (2 mg/kg/day) would give an approximated blood concentration of 0.025 ppm (25 ppb). McQueen et al (2012) recently evaluated glyphosate exposure to pregnant women and concluded that estimated exposures based on actual measurements in food were only 0.4% of the acceptable daily intake.

   The "Roundup" LC50 concentration used (57.5 ppm) is more than 2000-fold higher than the anticipated concentration (based on Anadon et al., 2009) following maximum allowable intake.

4) **Animal data and human experience contradicts findings of Petri dish experiments with glyphosate-surfactant herbicides**. Glyphosate has been tested extensively in higher order animals (Giesy et al., 2000; Williams et al., 2000). There is no evidence for developmental or reproductive concerns in multiple species despite numerous high-dose tests by different manufacturers (Williams et al 2012, EU, 2002, JMPR/WHO 2004). Furthermore, studies with POEA have not demonstrated any target organ toxicity or effects on embryos, fetuses, or the placenta (Williams et al., 2000; Williams et al 2012).

5) **The surfactant effects are not surprising.** Levine et al., 2007 demonstrated that surfactants found in household and personal care products could alter mouse Leydig cell function. It should not be a surprise that a glyphosate-based formulation which contains surfactants similar to surfactants found in household and personal care products would have an effect on cellular membranes. The exposure of humans to surfactants is common from bath gels, hand soaps, shampoos, and laundry and dishwashing detergents to name a few. In addition human oral exposure to surfactants can originate from residues on eating utensils and dishes washed with dish washing detergents and from residues taken up via drinking water (HERA, 2003).

6) **Doses of Cry proteins are irrelevant to real life exposures.** High-dose animal toxicity testing via the oral routes using Cry1Ab and Cry1Ac demonstrates no toxic effects at doses thousands of times higher than any potential human intake. The only concentration of Cry protein demonstrating any effect on cellular function was 100 ppm, used in an otherwise protein free medium. The concentration

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of Cry protein in grain is below 1 ppm (see Monsanto product safety data, link above), and these cry proteins are both degraded by cooking and are readily digestible.

Studies of meat, milk, and eggs have not demonstrated intact Cry protein detection in animals fed on GM crops containing these proteins. The studies of Aris and Leblanc, taken at face value, indicate Cry protein concentration in human blood up to about 0.2 parts per billion- or 500-fold LESS than the concentrations used by Mesnage et al.(2012). We would note, however, that the validity of the Aris and Leblanc (2011) publication has been seriously questioned by scientists and regulators. Regulatory opinions, original article, and associated correspondence at:
http://www.food.gov.uk/multimedia/pdfs/acnfp10308pest
http://www.foodstandards.gov.au/consumerinformation/gmfoods/fsanzresponsetostudy5185.cfm
http://www.sciencedirect.com/science/article/pii/S0890623811000566 .

7) **The co-application of Cry protein with the glyphosate-surfactant REDUCES the apparent degree of cellular injury (as measured by induction of caspase levels).** This occurs even at concentrations of Cry1Ab which the authors report to cause cellular injury and membrane disruption. This is worth noting for several reasons:

   First, it brings into question the toxicity observations with Cry1Ab, as the argument that membrane disruption and impaired mitochondrial function should be protective seems to be highly untenable, especially in view of the studies (Levine et al. 2007) demonstrating the mitochondrial membrane activity of surfactants.

   Second, it should take off the table any implications of a "synergistic effect" of Cry proteins and glyphosate-surfactant herbicides. (The direction is, if anything, antagonistic, but we would not argue for any true in-vivo protective effects as the entire system is fundamentally irrelevant.)

   Third, this probably is demonstrating the artificiality of the system itself. As noted above, this is a protein-free medium. Protein protects cells in culture by multiple mechanisms- binding to toxic materials, binding to potential receptor sites, or other non-specific surface-stabilization effects. It appears from Mesnage's own data that simple addition of protein to their system, even at low concentrations (and even if that protein is a Cry protein) protects from toxicity.

8) **The references cited regarding the in-vitro toxicity studies of other Bt derived proteins are largely irrelevant.** There are many different Bt varieties that produce many different kind of toxins, and some Bt toxins are known to be toxic to mammalian cells when in vitro.We utilize the Cry proteins that are closely related to the many kinds of proteins found in commercial Bt microbial pesticides that have been safely used in agriculture around the world for approximately 50 years. The Bt toxins used in GM planta have been subject to extensive safety assessment (Betz 2000, Federici and Siegel 2008, OECD 2007, WHO/IPCS 1999)The work of Ito et al reports the effect of a NON-insecticidal Bt-derived protein which is cytotoxic to some human cell lines. The work of Nagamatsu similarly reports on a non-insecticidal Bt protein. The work of Rani does involve a solubilized Cry protein from an insecticidal Bt strain. Oral toxicity is not demonstrated in any case but, more to the point, extensive toxicity studies of Cry1Ab and Cry1Ac in mammalian species indicate to toxic effect at relevant doses and by relevant routes.

9) **Caffeine metabolites, alcohol and nicotine can disrupt cell function.** It is important to note that a metabolite of caffeine inhibited the development of Leydig cells in Petri dish experiments. (Pollard et al., 2001). In addition, alcohol (Jang et al., 2002) and nicotine (Kim et al., 2005) activate specific intracellular death-related pathways, capsase -3, inducing apoptosis in mouse Leydig cells grown in Petri dishes similar to that reported in this abstract. *In vivo* and *in vitro* exposures demonstrate that alcohol can damage Sertoli cells (Shu et al 1997). These findings clearly put this experimental model into context. Caffeine, in its natural and added forms, is found in coffee, tea, cola beverages, energy drinks, chocolate and even some medicines. The average intake of caffeine in the US by children 5-18 years of age averages 1 mg/kg/day and adults 2.4 mg/kg/day (Mandel, 2002). A typical cup of coffee

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can contain 150 mg of caffeine, a cup of blended tea 43 mg and a small portion of a milk chocolate candy bar contains about 7 mg of caffeine. (Health Canada 2010).

10) **Prior Publications.** Seralini and colleagues at the University of Caen in France have five prior publications on the results of exposing unprotected cells in culture to glyphosate, AMPA (aminomethylphosphonic acid, the primary environmental degradate of glyphosate), glyphosate- based formulations or a surfactant used in some formulated products.

- Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., and Seralini, G.-E. 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ. Health Perspect. 113:716-720. http://ehp03.niehs.nih.gov/article/fetchArticle.action?articleURI=info:doi/10.1289/ehp.7728

- Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., and Séralini, G. E. 2007. Time- and dose-dependent effects of Roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53:126-133. http://www.springerlink.com/content/d13171q7k863l446/

- Benachour, N., and Séralini, G. E. 2009 Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells. Nora Benachour and Gilles-Eric Seralini. Chem. Res. Toxicol., 22, 97–105. http://pubs.acs.org/doi/pdf/10.1021/tx800218n

- Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M., Gilles-Eric Seralini (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology; 262(3):184-91 http://www.sciencedirect.com/science/article/pii/S0300483X09003047

- Clair E, Mesnage R, Travert C, Séralini GE., 2012. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicology in Vitro. http://www.sciencedirect.com/science/article/pii/S0887233311003341

The same group has published two publications suggesting that homeopathic remedies can protect cells against purported adverse effects of glyphosate. Co-authors are associated with the purveyor of these homeopathic products, although they claim no conflict of interest.

- Gasnier et al. Dig1 protects against cell death provoked by glyphosate-based herbicides in human liver cell lines. Journal of Occupational Medicine and Toxicology 2010, 5:29 http://www.occup-med.com/content/5/1/29

- Gasnier et al. Defined plant extracts can protect human cells against combined xenobiotic effects. Journal of Occupational Medicine and Toxicology 2011, 6:3 http://www.occup-med.com/content/6/1/3

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EU Commission. (2002) Review report for the active substance glyphosate. http://ec.europa.eu/food/plant/protection/evaluation/existactive/list1_glyphosate_en.pdf

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Health Canada. http://www.hc-sc.gc.ca/hl-vs/iyh-vsv/food-aliment/caffeine-eng.php

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Author(s)	Year	Study title
Clair E., Linn, L., Travert, C., Amiel, C., Seralini, G.E	2012b	Effects of Roundup® and Glyphosate on Three Food Microorganisms: *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus* Current Microbiology Volume: 64 Number: 5 Pages: 486-491

Abstract*

Use of many pesticide products poses the problem of their effects on environment and health. Amongst them, the effects of glyphosate with its adjuvants and its by-products are regularly discussed. The aim of the present study was to shed light on the real impact on biodiversity and ecosystems of Roundup®, a major herbicide used worldwide, and the glyphosate it contains, by the study of their effects on growth and viability of microbial models, namely, on three food microorganisms (*Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) widely used as starters in traditional and industrial dairy technologies. The presented results evidence that Roundup® has an inhibitory effect on microbial growth and a microbicide effect at lower concentrations than those recommended in agriculture. Interestingly, glyphosate at these levels has no significant effect on the three studied microorganisms. Our work is consistent with previous studies which demonstrated that the toxic effect of glyphosate was amplified by its formulation adjuvants on different human cells and other eukaryotic models. Moreover, these results should be considered in the understanding of the loss of microbiodiversity and microbial concentration observed in raw milk for many years.

* Quoted from article

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MATERIALS AND METHODS

1. Test material:

Test item:	Roundup® R400 and R450
Active substance(s):	Glyphosate
Description:	Not reported
Source:	Roundup® R400 and R450:Monsanto, Anvers, Belgium Glyphosate: Sigma-Aldrich, France
Lot/Batch #:	Not reported
Purity:	400 g/L and 450 g/L glyphosate

2. Test system/cells:

Species:	*Geotrichum candidum* ATCC 204307
Test system:	Fungi
Source:	Not reported
Maintenance medium:	MSF, pH 5.6
Initial cell concentration:	$1.95 \times 10^4 \pm 0.36$ UFC/mL

Species:	*Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1

Test system: Bacteria
Source: Not reported
Maintenance medium: M17 containing lactose, pH 7.1
Initial cell concentration: $6.31 \times 10^5 \pm 2.03$ UFC/mL

Species: *Lactobacillus lactis* subsp. *cremoris* ATCC 19257
Test system: Bacteria
Source: Not reported
Maintenance medium: MRS, pH 6.4
Initial cell concentration: $6.70 \times 10^8 \pm 2.52$ UFC/mL

3. Cell treatments:

Exposure: Roundup R400 (400 g/L of glyphosate) and R450 (450 g/L of glyphosate) were diluted in autoclaved culture media, pH adjusted to each medium and 0.2 µm filtered. A solution of glyphosate, equivalent in glyphosate concentration and pH to R450, was diluted in different media.

The minimal inhibitory concentration (MIC) was evaluated, after treatment, by turbidimetry measurement at 600 nm using a microplate reader. The minimal microbicide concentration (MMC) which corresponds to the minimal treatment leading to 99.90% of lethality was evaluated by colony counting, after plating the previously treated micro-organisms. Concentrations between the MIC value and the MMC value correspond to cells that do not growth but are not dead.

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4. Observations:

Colonies observations: Each microorganism was plated on Petri dishes containing agar and Roundup, and then incubated during 48 h. Colonies were macro- and microscopically observed after coloration with cotton blue for *G. candidum* and methylene blue for the two bacteria.

Statistics: Data were expressed as mean ± SEM for three independent determinations (n = 9). Significant differences were determined by Student *t* test with P<0.05*, P<0.01** and P<0.001***.

KLIMISCH EVALUATION

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

Comment: Non-validated, non-guideline test with methodological and reporting deficiencies (e.g. dose concentrations in media not specified, positive controls or controls that show the validity of the test system /and concentration range tested). Inappropriate test system for formulations containing surfactant; cytoxic membrane disruption potential of surfactants are well known for in vitro test systems.

2. Relevance of study: Not relevant (Due to reliability)

3. Klimisch code: 3

GTF Comments

- Clair et al look at in vitro effects of Roundup and glyphosate on *Geotrichum candidum* (a yeast-like fungal species found in some ripening cheeses) and two lactobacilli - *Lactococcus lactis* and *Lactobacillus delbrueckii*.
- The authors fail to provide any evidence whatever of difficulties related to the presence/ absence/ population of these organisms in dairy production or in the environment.
- Glyphosate at 1% had no effect on lactobacilli but did impair *Geotrichum*, which is unsurprising as glyphosate at herbicidal concentrations will impact metabolism of many fungi, which (like plants) use the shikimate pathway for aromatic amino acid production.
- At concentrations at or above 100 ppm glyphosate-which would be equivalent to surfactant concentrations around 30 ppm and up (approximate, as the precise formulations are not known), one sees growth inhibition of all 3 tested species.
- Surfactants are known to be bacteriostatic, with (for example) quaternary ammonium compounds typically being active in the 30-150 ppm range.
- A wide variety of disinfectants, including surfactants, are approved and recommended for use in food services, including milk processing. The surfactant based products typically provide concentrations (at use dilution) of 50-500 ppm.
- General recommendations for liquid milk processing facilities generally recommend daily cleaning using a disinfectant solution. Cleaning products generally contain surfactants as milk residues are fat-containing and thus difficult to remove with water alone.
- Glyphosate residues in milk are permitted - currently at 0.1 ppm in the United States. This is 100,000 times LESS than the only effective concentration tested (10,000 ppm) for glyphosate.
- The results of this study have no apparent applicability to effects of glyphosate-surfactant herbicides on soil microbial populations.
- In short, Clair et al demonstrate that surfactants are bacteriostatic for these 3 organisms at concentration ranges well within the range of concentrations generally found to be useful for sanitation purposes. Application dilutions (1%) of glyphosate were shown to inhibit a yeast-like organism, which is unsurprising. Surfactant solutions are routinely used to sanitize food processing equipment at concentrations at or above those tested by Clair et al. These concentrations are vastly higher than the concentrations of glyphosate or possible surfactant present (if any) in incoming milk. This paper is simply irrelevant to agricultural use of glyphosate.
- Surfactants kill micro-organisms. This research is a valuable reminder of the importance of washing our hands before we eat.

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Author(s)	Year	Study title
Williams, G.M. Kroes, R. Munro, I.C.	2000	Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for humans. Regulatory Toxicology and Pharmacology Volume: 31 Pages: 117-165

Abstract*

Reviews on the safety of glyphosate and Roundup herbicide that have been conducted by several regulatory agencies and scientific institutions worldwide have concluded that there is no indication of any human health concern. Nevertheless, questions regarding their safety are periodically raised. This review was undertaken to produce a current and comprehensive safety evaluation and risk assessment for humans. It includes assessments of glyphosate, its major breakdown product [aminomethylphosphonic acid (AMPA)], its Roundup formulations, and the predominant surfactant [polyethoxylated tallow amine (POEA)] used in Roundup formulations worldwide. The studies evaluated in this review included those performed for regulatory purposes as well as published research reports. The oral absorption of glyphosate and AMPA is low, and both materials are eliminated essentially unmetabolized. Dermal penetration studies with Roundup showed very low absorption. Experimental evidence has shown that neither glyphosate nor AMPA bioaccumulates in any animal tissue. No significant toxicity occurred in acute, subchronic, and chronic studies. Direct ocular exposure to the concentrated Roundup formulation can result in transient: irritation, while normal spray dilutions cause, at most, only minimal effects. The genotoxicity data for glyphosate and Roundup were assessed using a weight-of-evidence approach and standard evaluation criteria. There was no convincing evidence for direct DNA damage in vitro or in vivo and it was concluded that Roundup and its components do not pose a risk for the production of heritable/somatic mutations in humans. Multiple lifetime feeding studies have failed to demonstrate any tumorigenic potential for glyphosate. Accordingly, it was concluded that glyphosate is noncarcinogenic. Glyphosate, AMPA, and POEA were not teratogenic or developmentally toxic. There were no effects on fertility or reproductive parameters in two multigeneration reproduction studies with glyphosate. Likewise there were no adverse effects in reproductive tissues from animals treated with glyphosate, AMPA, or POEA in chronic and/or subchronic studies. Results from standard studies with these materials also failed to show any effects indicative of endocrine modulation. Therefore, it is concluded that the use of Roundup herbicide does not result in adverse effects on development, reproduction, or endocrine systems in humans and other mammals. For purposes of risk assessment, no-observed-adverse-effect levels (NOAELs) were identified for all subchronic, chronic, developmental, and reproduction studies with glyphosate, AMPA, and POEA. Margins-of-exposure for chronic risk were calculated for each compound by dividing the lowest applicable NOAEL by worst-case estimates of chronic exposure. Acute risks were assessed by comparison of oral LD50 values to estimated maximum acute human exposure. It was concluded that, under present and expected conditions of use, Roundup herbicide does not pose a health risk to humans.

* Quoted from article

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1. Test materials addressed:

Test item:	Roundup®
Active substance(s):	Glyphosate (as isopropylamine salt)
Concentration::	356 g glyphosate free acid equivalent/L (480 g/L salt)

Test item:	Glyphosate
Purity:	96 %

Test item: POEA (polyethoxylated tallow amine)

Test item: AMPA (aminomethylphosphonic acid)

2. Studies addressed:

Metabolism and pharmacokinetics: With Glyphosate, AMPA, and Roundup

Glyphosate oral dosage studies in rats

- Absorption;
- Tissue distribution;
- Biotransformation/Excretion;

AMPA single oral dose study in rats

Glyphosate/AMPA oral studies in non-rodents

Glyphosate and Roundup – Dermal penetration

Toxicology studies: With Glyphosate and AMPA

Acute toxicity and irritation studies

Subchronic toxicity studies (glyphosate – mouse, rat, dog; AMPA – rat and dog)

Chronic toxicity/Oncogenicity studies (glyphosate – mouse and rat; AMPA – rat)

Reproduction/Developmental toxicology studies

Toxicology studies: With POEA and Roundup

Acute toxicity and irritation studies

Subchronic toxicity studies (POEA – rat and dog; Roundup – rats)

Reproduction/Developmental toxicology studies

Genetic toxicology studies: With Glyphosate, formulations, and AMPA

Gene mutation studies (bacteria, mammalian cells, *D. melanogaster*)

Chromosomal aberration studies

In vitro sister chromatid exchange

In vivo micronucleus assay

In vivo mutation (dominant lethal test)

DNA damage/reactivity (UDS, rec-assay, DNA adducts, alkaline elution)

Mutation studies with AMPA

Evaluation of potential specific organ/system effects:

Salivary gland changes

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Potential for endocrine modulation (*in vitro*; *in vivo*)
Potential for Neurotoxicity
Potential for synergistic interactions

Human experience:

Irritation studies
Occupational exposure
Effects observed after ingestion

Exposure assessment:

Dietary exposure to residues in food (glyphosate, AMPA, and POEA)
Occupational dermal and inhalation exposure during application (AMPA and POEA)
Non-occupational exposure during application
Consumption of water (glyphosate, AMPA, and POEA)
Reentry into treated areas (glyphosate, AMPA, and POEA)
Bystander exposure during application
Possible inadvertent exposures derived from specific activities
Aggregate exposure estimates

Risk characterization: For Glyphosate, AMPA, and POEA

Identification of LD_{50}s, NOAELs
Derivation of the MOE, estimation of risks to Humans from acute or chronic exposure

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KLIMISCH EVALUATION

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

Comment: The publication represents an expert consideration on safety evaluation and risk assessment of the compounds, performed according to current scientific principles.

Available toxicity studies are summarized, but the generation of original data is not subject of the present publication.

2. Relevance of study: **Review of major relevance for the safety evaluation of glyphosate.**

3. Klimisch code: **Not applicable**

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IIA 5.11 Summary of mammalian toxicity and overall evaluation

Toxicokinetics and metabolism

Absorption, distribution and elimination

The 2001 EU evaluation of glyphosate concluded that following oral administration, glyphosate is rapidly absorbed from the gastrointestinal tract but only to a limited extent of approximately 30 - 40 %. Data on the extent of biliary excretion in bile-cannulated rats, that was not available for the 2001 EU glyphosate evaluation, confirms the systemically available glyphosate is excreted exclusively in the urine (1996). Elimination of ingested glyphosate via faeces and systemic glyphosate via urine is rapid and is nearly complete within 48 hours. The pulmonary route of elimination is negligible (< 0.2%, 1995 and 1996). Faeces contain unabsorbed glyphosate.
Distribution into the organs and tissues after an oral dose is rapid but limited with generally low residues found in organs and tissues at termination. After a period of 3 to 7 days following oral administration, total body burden accounted for less than 1% of the applied radioactivity. There is no evidence of a potential for accumulation in animals based on residue analysis in organs and tissues after 72h -168 h. Elimination from bone is slower than from other tissues. However, the amount of radiolabel in bone after 168h after a single oral dose was relatively low at 0.02 – 0.03% of the applied dose (1995). The highest residues were measured in bone, followed by kidney and liver. This pattern of absorption, distribution and elimination was not significantly changed either by single high doses administered or by repeated administration of low doses. Similarly, the sex of the test animals did not affect the results. The pattern of distribution of radioactivity in whole-body autoradiograms showed the greatest intensity of radioactivity to be in bone and gastrointestinal tract at up to 24 hours after dosing which was reduced to negligible amounts within 48 hours (1996). Peak plasma levels were observed within 4 – 6 h and elimination from blood and plasma was rapid with no evidence of accumulation in blood cells. A biphasic pattern of elimination of radiolabel in plasma has been suggested from the plasma radiolabel in a range of studies and terminal half lives have been estimated at 8 – 10h. Radiolabel in plasma was negligible after 24 h and not detected at 168 h.

Metabolism

Metabolism of glyphosate is very limited. Most of the parent glyphosate is eliminated unchanged and a small amount, just under 0.5% of the applied dose is eliminated as aminomethylphosphonic acid (AMPA). While glyphosate is known to be the major metabolite of glyphosate in plants, metabolism in mammals has been shown to be very limited.

Acute toxicity

The 2001 EU evaluation of glyphosate concluded that glyphosate acid and its salts exhibit a low acute toxicity in laboratory animals by the oral and dermal route with LD_{50} values greater than 2000 mg/kg bw in previously conducted studies. These results were confirmed in other and new studies recently performed since the last review. Given all LD_{50} values exceed the highest dose tested and differences between the EU and GHS classification criteria, the acute oral and dermal toxicity endpoints should be amended to greater than 5000 mg/kg bw/day.
Glyphosate acid is of low acute inhalation toxicity with LC_{50} values above the limit test dose of 5 mg/L air per 4 hours obtained for the acid and the isopropylammonium salt (IPA). The ammonium salt was tested up to the maximum attaninable concentration of 1.9 mg/L with comparable clinical signs but no deaths occurring. Achieving inhalation doses up to the limit dose of 5 mg/L were often a technical challenge, based on the physic-chemical properties the test materials.
Regarding primary irritation, glyphosate acid and the salts were found to be non-irritant to intact skin and only slightly irritant to abraded skin. Studies conducted since the last EU review have confirmed that glyphosate acid is either non-irritating or only slightly irritating to the skin and that no classification is required.
Glyphosate acid was found to be strongly irritating to rabbit eyes requiring classification; previously as R41 – **'Risk of serious damage to eyes'** and now **'Irreversible effects on the eye/serious damage to eyes (Category 1)'** under GHS. Recently performed studies on the eye irritating potential of glyphosate acid supported the previous findings and classification. There was markedly less eye irritation observed

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with the salts which are used in formulated products, presumably due to the salts having a more neutral pH than glyphosate acid.
Glyphosate acid has been tested for skin sensitisation by the stringent Magnusson-Kligman test method and the Buehler test method in guinea pigs. Glyphosate acid has also been tested for its skin sensitising properties in the local lymph node assay in mice. In all study types glyphosate acid was unequivocally negative for skin sensitisation potential. In addition, the IPA salt tested negative for skin sensitisation potential in a Magnusson-Kligman test in guinea pigs.

Short-term toxicity
Sub-acute and sub-chronic toxicity studies note a low oral toxicity of glyphosate and its salts in rats, mice and dogs.

Rodents:
In rats, the previous 2001 EU glyphosate evaluation concluded that the lowest NOEL was about 100 mg/kg bw/day in 90-day feeding studies in rats with the first effects occurring in the range 250-300 mg/kg bw/day, however in most studies higher NOAELs were established. Liver effects were observed indicated by clinical chemistry and organ weight changes in rats. Soft stools and diarrhoea, together with occasionally reduced bodyweight gain and food consumption, suggest irritation of the gastrointestinal tract at high dose levels. In some oral rat studies cellular alterations in salivary glands were observed upon histopathological examination. The glyphosate taskforce believes these salivary gland findings are a non-adverse adaptive response to treatment with a low pH diet. Overall the mouse is less sensitive than the rat with only effects observed on body weight at very high dose levels. Additional studies included in this submission that have not been previously reviewed also demonstrate that glyphosate is of low oral toxicity. The NOAELs ranged between 79-765 mg/kg bw/day and the lowest LOAEL observed in rats was 569 mg/kg bw/day ([redacted] 1995). Consistent with the previously reviewed studies effects were observed on clinical chemistry parameters (often non-specific markes of mild toxicity), bodyweight gain and food consumption at high dose levels. Additionally, caecum distension and an increase in caecum weight were observed in both a 13 week rat study ([redacted] 1995) and a 13 week mouse study ([redacted] 1995) . This effect appeared to be dose related at very high dose levels in both species but was not assocated with any corollary histopathological changes and is therefore of uncertain toxicological relevance. In contrast, in another 13 week dietary rat study ([redacted] 1996) mucosal atrophy of the caecum was observed where there was no associated weight change. Another finding observed in male mice that had been previously reported was cystitis of the urinary bladder in animals dosed at 6295 mg/kg bw/d.
Overall the NOEL/NOAEL levels established in the 90-day dietary studies in rats varied between approximately 80 and 1600 mg/kg bw/d. The lowest effect dose was in the range of 550 mg/kg bw day ([redacted] 1995). Mice appeared to be less sensitive, with substantially higher NOAEL values, the lowest of which was 600 mg/kg bw/d.

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Dogs:
In oral sub-chronic toxicity studies in the dog previously evaluated in the 2001 EU glyphosate evaluation, only unspecific signs of toxicity (decrease in body weight gain and food consumption) were observed at high dose levels. In two dietary dog studies performed at the same laboratory, liver effects of equivocal toxicological significance were observed at low doses (8-29 mg/kg bw/d). However, the previous evaluation found that because these findings were not confirmed in more recent studies using much higher dose levels they were not considered to be compound-related. The previous review concluded the lowest relevant NOAEL was 300 mg/kg bw/d for glyphosate acid and the IPA salt.
This NOAEL is supported by four recently conducted studies ([redacted], 2007, [redacted] 1999, [redacted] 1996 [redacted] 1996). In the [redacted] (1999), [redacted] (1996) and [redacted] (1996) studies only minor effects on bodyweight and clinical chemistry parameters were observed at 1000 mg/kg bw/day. In the [redacted] (2007) study at 1000 mg/kg bw/day the test item administration induced marked clinical signs and mortality leading to the early termination of the group at week 11, however, the 300 mg/kg bw/d was the NOAEL.
Overall the lowest NOAEL observed was 253 mg/kg bw/d and the highest was 1000 mg/kg bw/day. The lowest effect level was 1000 mg/kg bw/d in 90 day dog studies.

Oral one year toxicity in the dog was previously evaluated in the 2001 EU glyphosate review. The (1991) and and (1985) studies have been previously evaluated and like the 90-day studies only non-specific signs of toxicity (slight effect body weight and an increase in clinical signs of soft, liquid stools) were observed at limit dose. The previous review concluded the lowest relevant NOAEL was 300 mg/kg bw/d for glyphosate acid. These previous conclusions were confirmed by 3 additional one year dog studies. Again non-specific effects of toxicity were observed at doses at, or close to limit dose. These effects were characterised as reduction in body weight gain, reduction in urinary pH and minor effects on clinical pathology parameters. The lowest dose level where treatment related effects were observed was 926 mg/kg bw/day in the (1996) study. The most relevant one year oral dog NOAEL for glyphosate technical is 500 mg/kg bw/day.

One study on sub-acute inhalative toxicity (14 days) in rodents (1985: non-GLP (pre-GLP) study) was reviewed in the 2001 EU evaluation. The previous review concluded no treatment-related effects were observed and the NOEL was 3.8 mg/L. No further studies have been conducted.

The short-term percutaneous toxicity of glyphosate has been investigated in the rat and rabbit. In both Sprague-Dawley (SD) (1993) and Wistar derived (1996) rats no signs of systemic toxicity were noted following dosing for 21 days at 1000 mg/kg bw/day the limit dose for this study type. Three studies were conducted in New Zealand White rabbits ( ) and doses ranged from 1000 mg/kg bw/day to 5000 mg/kg bw/day. In both the rat and the rabbit no signs of systemic toxicity were evident following repeated application of glyphosate to the skin. The NOAEL for short term percutaneous toxicity was 1000 mg/kg bw/day in the rat and 5000 mg/kg bw/day in the rabbit as previously concluded inthe 2001 EU glyphosate evaluation.

Genotoxicity

In the 2001 EU evaluation glyphosate was examined for mutagenicity and clastogenicity in a wide range of test systems covering all relevant endpoints *in vitro*. Additional studies have been conducted on glyphosate since the last EU review, however, all these studies were negative and are considered confirmatory data. Glyphosate has clearly been proved to have no genotoxcity potential a wide range of regulatory studies *in vitro*. During the 2001 EU glyphosate evaluation, a number of *in vivo* cytogenicity studies and bone marrow micronucleus tests in rats and mice have been evaluated. The last review concluded that glyphosate is not clastogenic *in vivo*. Since the last review the ability of glyphosate to cause chromosomal aberrations has been further investigated in the *in vivo* micronucleus test (2009b, 2007, 2008, 1999, 2006, 1996 2008). All new studies were considered negative. Glyphosate has been tested in a wide array of *in vitro* and *in vivo* genotoxicity assays. Overall, in the vast majority of studies performed, glyphosate proved clearly negative and it can be concluded that the active ingredient does not exhibit a genotoxic risk to humans.

Chronic toxicity and Carcinogenicity

The long-term toxicity and carcinogenic potential of glyphosate has been assessed in rats and mice. The 2001 EU glyphosate evaluation concluded that in long-term studies in rats and mice there was no evidence of carcinogenicity. It also concluded that in rats, there was no adverse effects on survival or clinical signs. A reduction in body weight gain, increases in alkaline phosphatase and liver weight changes, an increase in incidence of cataracts, inflammation of the gastric mucosa and histopathological changes in the salivary glands were observed sporadically across the studies previously reviewed. In the mouse the previous 2001 review concluded that non-neoplastic treatment related effects were limited to high dose males in the (1983) study and comprised of a reduction in body weight gain, hepatocyte hypertrophy and bladder epithelial hyperplasia.
Five additional long term studies have been conducted in the rat and 3 in the mouse that have not been previously reviewed at the EU level. There was no evidence that glyphosate acid is carcinogenic in any of these studies that have not been previously submitted.

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Rat:

A 1-year toxicity study (1996) was performed in rats with dietary doses of 0, 2000, 8000 and 20000 ppm glyphosate acid. Based on body weight and salivary gland effects at 20000 ppm, the NOAEL for toxicity for glyphosate acid was 560 mg/kg bw/day in males and 671 mg/kg bw/day in females.

In 2-year dietary rat study, by (1997), rats received diets providing 0, 3000, 10000 or 30000 ppm glyphosate. The NOAEL for toxicity is 104 and 115 mg/kg bw/day for males and females, respectively, based on histopathological and clinical effects of the caecum together with follicular hyperkeratosis and/or folliculitis/follicular abscess in the mid and high dose groups.

In another combined chronic toxicity and carcinogenicity study (2001) which was performed with glyphosate technical in rats receiving diets providing 0, 2000, 6000 or 20000 ppm glyphosate acid, the NOAEL was set at 361 and 437 mg/kg bw/day for males and females, respectively. It was based on liver and kidney effects, prostatitis, periodontal inflammation, urinary acidosis and haematuria, which may be attributed to the acidity of the test substance.

The 2 year dietary rat study conducted by (1997) concluded that there were no adverse treatment related effects and the NOAEL was 1290/1740 mg/kg bw/day in males and females respectively.

The most recent rat dietary carcinogenicity study was conducted in 2009 by , again there were no adverse treatment related effects at the highest dose tested. The NOAEL for this study was 1230 mg/kg bw/day.

In the previous review salivary glands have been suggested as possible target organ. Histological changes described as "cellular alteration" in the parotid and mandibular salivary glands and a higher organ weight of these glands were noted at 100 mg/kg bw/day and higher (1993). These findings determined the lowest NOAEL in the previous review from the long term studies. In addition similar changes have been observed in subchronic rat studies. In contrast, there are several chronic studies where no effects on the salivary glands were reported. These differences may be more or less pronounced depending on the rodent strain used or methodological differences. Additional studies were conducted to examine species sensitivity, reversibility of the effects and the hypothesis previously suggested in the WHO/FAO 2004 evaluation of glyphosate; that local irritation of the oral cavity by the organic acid mixed into diet may result in an adaptive salivary gland response (IIA 5.10). Based on the outcome of these examinations the treatment-related pathological findings (increased salivary gland size and flow) can be considered as adaptive responses due to oral irritation from the ingestion of glyphosate acid in the diet. When the salivary glands are viewed in perspective as an adaptive change, the lowest effect level in the long-term rat studies is 354/393 mg/kg bw/day in males and females respectively (1997). Overall the NOEL/NOAEL levels established in the long term studies in rats varied between approximately 31 mg/kg/day (300 ppm in diet, the highest dose tested in this pre-guideline study, considered a supplementary study in the EU monograph) and 1740 mg/kg bw/day.

Mouse:

A combined toxicity and carcinogenicity stude in mice (2001, 5.5.3/01) demonstrated a slightly higher mortality in the high dose group. Mortality was within the upper end of the the historical control range. However, treatment with glyphosate might slightly have affected the mortality at the highest dose of 10000 ppm, and because a relationship to treatment was unclear a conservative NOAEL for toxicity at the mid dose of 1000 ppm (150.5 mg/kg bw/day for combined sexes) was set for this study. The number of malignant lymphoma, the most common tumour in the mouse, was slightly elevated in the high dose group compared to control, but this was considered as incidental background variation based on historical control data and was not considered to be related to treatment. However it should be noted that the high dose group received a daily achieved dose of 1460 mg/kg bw/day which is in excess of the limit dose recommended by most current international guidelines.

In the study by 1997 the low effect level was 8000 ppm (equivalent to 787 mg/kg bw/day) in females only based on a reduction in body weight gain. At the top dose of 40000 ppm (equivalent to 4348/4116 mg/kg bw/day in males and females respectively) signs of toxicity included loose stools, reduced body weight gain, food consumption and food utilisation, caecum distention and increased absolute and relative caecum weight (without corollary histopathological findings), increased incidence of anal prolapsed consitent with histopathological erosion/ulceration of the anus.

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The most recent 80-Week dietary mouse study was conducted by 2009. There were no adverse treatment related effects at the highest dose tested. The NOAEL for this study was 810/1081 mg/kg bw/day in males and females respectively.
Overall the lowest effect level observed in the long-term mouse studies was 787 mg/kg bw/day in females in the (1997) study and the NOEL/NOAELs ranged from 151 – 1081 mg/kg bw/day.

There was no evidence for a carcinogenic potential of glyphosate noted in any of the studies performed in rats and mice.

Reproductive Toxicity

In the 2001 EU glyphosate evaluation a number of multigeneration studies were reviewed. It was concluded that glyphosate acid did not indicate a specific hazard for reproduction. It concluded that weak effects on the offspring as evidenced by reduced pup weight were confined to high dose levels where compound related effects were observed in the parent animals. Since the last review three additional studies have been conducted. Study summaries are available for these new studies below.
In the first additional study by (1997) parental toxicity was evident at doses of 30000 ppm and consisted of reduced body weight, soft stool and distension of the caecum which was consistent with findings in the sub-chronic and chronic rats studies conducted at this laboratory. In this study, effects in offspring consisted mainly of reduced body weight and distension of the caecum at 30000 ppm only.
In the (2000) study the only effect of treatment was a reduction in the bodyweight of the F1A pups in the 10000 ppm group (1063/1634 mg/kg bw/day in males and females respectively) with a subsequent reduction in bodyweight of the selected F1 parent males for the duration of the mating period. The fertility and reproductive performance of each generation of parental animals and the clinical condition and survival of their offspring were not adversely affected by treatment.
In the most modern study by (2007) there was no treatment-related effects on reproductive performance, parents or offspring.
In the previously reviewed study (1992) there were minimal histopathological changes on the salivary glands in parental and offspring animals noted at the highest dose (i.e., 10000 ppm) and to a lower extent at the mid-dose (i.e. 3000 ppm). This observation was also noted in other repeated dose studies with glyphosate but is considered an adaptive response to high dietary doses of glyphosate, which is a strong organic acid, and can therefore cause irritation of the oral cavity leading to increased salivary excretion (see chapter IIA 5.10). Overall the lowest effect level for parental toxicity was 668-771 & 752-841 mg/kg bw/day in males and females respectively based on slightly reduced body weight in F1 males, increased food and water consumption F1 females in the (1992) study The relevant parental NOEL/NOAELs ranged from 197-1063 from 197-1063 mg/kg bw/day for males and 226-1634 mg/kg bw/day for females.
There were no effects on reproduction (reproductive performance, fertility, parturition, lactation, sperm parameters and oestrus cycle) noted in any of the dose groups in any of the studies.
The lowest effect level for the offspring was 1063/1634 mg/kg bw/day in males and females respectively based on reduced body weight of first generation pups during lactation. The relevant NOEL/NOAELs for reproductive toxicity ranged from 197-1063 mg/kg bw/day for males and 226-1634 mg/kg bw/day for females.

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Developmental Toxicity

The previous 2001 EU glyphosate review concluded that in the rat the lowest relevant NOEL for both maternal and developmental effects was 300 mg/kg bw/day and the lowest effect level was 1000 mg/kg bw/day. The evaluation found there was no evidence of teratogenicity. Two additional tetratogenicity studies have been performed in rats that have not been previously reviewed in the 2001 EU glyphosate evaluation. These studies are considered to be confirmatory data. Overall the lowest effect level for maternal and foetotoxicity was 1000 mg/kg bw/day and the appropriate overall NOAEL was 500 mg/kg bw/day for both the dams and the foetuses based on the 1996.

In rabbits the previous 2001 EU glyphosate review concluded that the NOEL for developmental effects was 350 mg/kg bw/day (1980) and that effects on the foetuses were only observed in the presence of marked maternal toxicity. Overall the previous evaluation determined that glyphosate was not

teratogenic in rabbits. Three additional studies have been included in this submission. The results from these studies are consistent with the data that has been previously reviewed, the pattern of maternal toxicity is consistent and effects on the foetuses were only observed in the presence of maternal toxicity.

In rabbits, glyphosate exposure via oral gavage led to clinical signs of toxicity in dams consistent with gastro-intestinal disturbances. Rabbits were more sensitive to oral gavage dosing than other species. Clinical signs observed included diarrhoea/soft faeces, reduced faecal output, reduced body weights, reduced food consumption and increased mortality. Overall maternal toxicity was observed at dose levels of 150 mg/kg bw/day and above. The highest relevant NOAEL for maternal toxicity was 100 mg/kg bw/day.

Foetotoxicity/developmental toxicity occurred at doses that were above (or rarely at the same dose as) the dose that caused maternal toxicity. Most indications of developmental toxicity were reduced ossifications of skull, phalangeal and sternebral bones, which are typically seen in the litters of pregnant animals that do not eat well and lose weight during pregnancy. The importance of this observation should not be misconstrued to mean that maternal toxicity in those cases was the proximate agent that injured the fetus, but rather that if exposures to the causative agent are kept below the doses that cause maternal toxicity, the developing offspring are protected. The lowest observed effects on the foetuses occurred at 300 mg/kg bw/day and were characterized by delayed ossification and decreased foetal weights (███ 1996). The relevant NOAEL for foetotoxicity is 250 mg/kg bw/day.

A report from an independent source (███ 2011) has claimed that congenital malformations, especially of the cardiovascular system, were caused by glyphosate exposure in this same series of studies. A variety of malformation was reported across the database of glyphosate studies, these included:

- Dilated aorta/narrow pulmonary artery
- Narrow aorta/dilated pulmonary artery
- Interventricular septal defect
- Cardiomegaly
- Single ventricle
- Retro-esophageal right subclavian artery
- Interrupted aorta
- Right subclavian artery arising from aortic arch
- "Seal-shaped" heart

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If glyphosate does cause congenital heart defects, it would be anticipated that the prevalence of congenital heart defects would be increased and one would expect the malformation rate to increase with increasing dose until the pregnant does would become intoxicated or the fetuses would die. The malformations occurred at a low incidence across all dose groups; they did not exhibit a positive dose-response; and often clusters of the malformations occurred in the same fetuses.

The incidence of aorticopulmonary septum-related defects in the combined control groups was 1/879 (0.1%); in the combined glyphosate-treated groups the incidence was 12/2250 (0.5%). One half of the malformed fetuses was found in litters exposed to the highest doses (450 and 500 mg/kg/day), which also experienced severe maternal toxicity including maternal deaths, abortions, and weight loss. If these groups are not considered because of the potential confounding factor introduced by maternal health issues, the incidence of the defects is 6/2049 (0.3%). These data show that the overall incidence of aorticopulmonary septum-related defects in offspring from mothers exposed to glyphosate at doses below those that cause severe maternal toxicity is similar to that seen in non-exposed rabbits.

The other prominent cardiovascular malformation is dilated heart. All observations of this finding (among both control and treated groups) occurred in a study conducted in a single laboratory (███ 1993). This study has several weaknesses including a small number of litters available for examination due to low pregnancy rates and maternal deaths in the mid- and high-dose groups. None of the other six studies reported dilated hearts, although there was a single case of cardiomegaly reported in the mid dose group of 100 mg/kg/day in the ███ (1993) study (this was considered not to be related to treatment). Neither the criteria used to diagnose dilated heart nor measurements of the hearts were provided, so it is not possible to directly compare the dilated heart findings to the hearts of the more than 2800 fetuses in the other

studies. It is possible that the observation of dilated hearts is due to overly stringent inspection compared to criteria used by other laboratories.

Taken together, overall data regarding potential cardiovascular malformations in the seven rabbit developmental toxicology studies do not support the contention that there is a clear compound related effect on the foetal heart.

Endocrine disruption potential

Glyphosate has been tested in a full battery of regulatory tests, including a number of rat reproduction (multigeneration), rat and rabbit developmental (teratology), one-year dog and lifetime rat studies. Such studies allow for the examination of toxicological effects following repeated exposure of a range of species to glyphosate.

In the reproduction studies, animals were exposed to the compound at all stages of their development; including adults prior to mating, developing animals in utero, offspring to maturity and repeated through a second generation. It is noteworthy that the most recent rat multigeneration study conformed to the 1998 US EPA and the new OECD 416 guidelines, and included a rigorous investigation of endocrine sensitive endpoints. These mammalian studies can be used to evaluate the ability of a test material to cause significant adverse effects through endocrine disruption.

The toxicological profile for glyphosate does not exhibit effects indicating endocrine disruption in mammals. No endocrine effect was noted in reproductive and developmental toxicity studies in rats or rabbits. Likewise, no effects on the endocrine system were observed in subchronic or chronic studies in rat, mouse and dog.

Neurotoxcity

The previous 2001 glyphosate evaluation concluded that there was no evidence of neurotoxicity in acute, subchronic or chronic studies in rodents and dogs. An acute neurotoxicity in rats was performed by (1996a) that was not previously reviewed during the 2001 glyphosate evaluation. Administration of glyphosate acid produced clinical signs of toxicity (including decreased activity, subdued behaviour, hunched posture, sides pinched in, tip-toe gait and/or hypothermia) at approximately 6 hours after dosing on day 1 in 3/10 females, only, which received 2000 mg/kg. One of these females was subsequently found dead on day 2. Quantitative assessment of landing foot splay, sensory perception, muscle weakness and locomotor activity revealed no changes indicative of neurotoxic potential. Histopathological evaluation of the central and peripheral nervous system revealed no treatment-related changes in animals receiving 2000 mg/kg. The no observed effect level (NOEL) for neurotoxicity, following single oral administration of glyphosate acid was 2000 mg/kg.

In addition a sub-chronic neurotoxicity study was also performed by (1996b). In this study administration of glyphosate acid produced no clinical signs of toxicity or effects on any of the quantitative functional observation battery tests or on locomotor activity that indicated any neurotoxic potential. In addition, there were no treatment-related changes in brain weight, length or width. Comprehensive histopathological evaluation of the peripheral and central nervous system revealed no evidence of any changes which could be attributed to administration of glyphosate acid. The no observed effect level (NOEL) for neurotoxic potential, following dietary administration of glyphosate acid for at least 90 days, was 20000 ppm (equivalent to 1547/1631 mg/kg bw/day in males and females respectively).

Other/special studies

From classical short- and long-term toxicity studies it was indicated that glyphosate possibly affects the salivary glands in rodents, which was described as increased basophilic staining and enlargement of cytoplasm especially in the parotid salivary glands. The toxicological significance of this effect was unexplained but assumed to be due to the low pH of the test substance blended into rodent diet. Therefore, a repeated feeding study in rats with citric acid was performed to evaluate the potential effects of the low pH on the salivary glands. Stastically significant higher parotid salivary gland weights and a stastically significant increase in severity of background cytoplasmic alterations in the parotid salivary glands were observed. In the absence of cytotoxicity and hyperplasia the noted effects were considered as an adaptive response rather than an adverse effect and are consistent with the hypothesis that low pH diets result in adaptive cellular responses within the salivary glands as similarly observed in studies with glyphosate

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2010). Furthermore, rat strain differences for this effect was investigated by (1996). Microscopic examination of the salivary glands showed the most pronounced effect occurred in the F344 rat strain where there was diffuse cytoplasmic basophilia and enlargement of the parotid acinar cells. Similar but slighter effects occurred in the AP and CD strains involving small foci of cells only. Based on the weight of evidence across the studies presented by the glyphosate taskforce it is proposed that the changes observed in the salivary gland (basophilia of the parotid acinar cells) are a non-adverse adaptive response to treatment with a low pH diet.

Pharmacological activity of the test substance was investigated *in vivo* with rats. No haematological, electrographic or behavioural/functional changes were observed 1996). In the same study, *ex vivo* investigations with isolated guinea pig ileum and isolated rat gastrocnemius muscle were performed. Glyphosate technical caused a contractile response similar to that seen with known parasympathomimetic agents on the isolated guinea pig ileum but did not cause any neuromuscular blocking activity on the innervated muscle.

In an immunotoxicity assessment repeated dietary administration of glyphosate to females B6C3F1 mice did not suppress the humoral component of the immune system. The no-observed-effect level (NOEL) for suppression of the humoral immune response in female B6C3F1 mice offered glyphosate in the diet for 28 days was considered to be 5000 ppm (equivalent to 1448 mg/kg bw/day), the highest dietary concentration.

Regarding the metabolite AMPA, studies conducted to evaluate the acute and sub-chronic toxicity, as well as mutagenicity and teratogenícty, demonstrate that AMPA possesses a lower toxicity than glyphosate.

Acute Reference Dose (ARfD)

Due to the low acute toxicity profile of glyphosate the derivation of an ARfD for glyphosate is not necessary for the following reasons:

- The mechanism of action for glyphosate herbicidal activity in green plants is not relevant to humans.
- Glyphosate is not acutely toxic; it did not produce mortality, overt clinical signs, changes in behaviour or relevant pathological lesions after a single dose up to 1000 mg/kg bw.
- No significant changes in clinical signs, behaviour, body weight and food consumption were observed in repeated-dose toxicity studies during the first few days with doses up to and above 500 mg/kg bw/day.

Acceptable daily Intake (ADI)

In the previous evaluation a chronic study was considered the most appropriate to derive the ADI. Since the rat proved the most sensitive species upon long-term exposure, it was suggested the ADI for glyphosate be based on the chronic toxicity data obtained in rats. The ADI was based on a NOAEL of 31/34 mg/kg bw/day (males/females) derived from a two year rat study. This was the highest dose tested in this study and animals at this dose showed no signs of treatement related toxicity. Since then, further chronic toxicity studies have been performed that indicate the appropriate NOAEL in long-term toxicity studies in rats is appreciably higher. An overview of the NOAELs and LOAELs observed in all chronic rat and mouse studies are presented in the following Table 5.11-1 and Figure 5.11-1 below.

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Table 5.11-1: Summary of long-term toxicity and carcinogenicity studies in rats and mice

	Reference (Owner**)	Type of study / Species	Dose levels (mg/kg bw/day)	NOAEL (NOAEL)* (mg/kg bw/day)	LOAEL (mg/kg bw/day) Targets / Main effects
Study not reviewed in the 2001 evaluation	IIA 5.5.1/01 1996 (SYN 1)	1-year, oral diet Rat, Wistar Alpk: AP_fSD	♂ 0, 141, 560, 1409 ♀ 0, 167, 671, 1664	560/671 ♂/♀ (1409/1664)	1409/1664 Salivary glands, body weight reduction
Studies from the 2001 evaluation	Annex B.5.5.1.2 Glyphosate Monograph IIA 5.5.2/01 1996 (FSG 1)	2-year, oral diet Rat, Wistar	0, 7.4, 74, 741 ♂ 0, 6.3, 59.4, 595 ♀ 0, 8.6, 88.5, 886	595/886 ♂/♀ 741 ♂+♀ (741 ♂+♀)	> 595/886 Only mild effects on clinical chemistry (liver enzymes) without histopathological changes
Studies not reviewed in the 2001 evaluation	IIA 5.5.2/02 1997 (ALS 1)	2-year, oral diet Rat, Sprague-Dawley	♂ 0, 104, 354, 1127 ♀ 0, 115, 393, 1247	104/115 (1127/1247)	354/393 Caecum weight increased, distension of caecum, loose stool, follicular hyperkeratosis and/or folliculitis/ follicular abscess, reduced body weight
	1997 (EXC)	2-year, oral diet Rat, Sprague-Dawley	♂ 0, 150, 780, 1290 ♀ 0, 210, 1060, 1740	1290/1740 (1290/1740)	> 1290/1740 Only mild toxic effects without histopathological changes
	IIA 5.5.2/03 2001 (SYN 2)	2-year, oral diet Rat, Wistar Alpk: AP_fSD	♂ 0, 121, 361, 1214 ♀ 0, 145, 437, 1498	361/437 (1214/1498)	1214/1498 Kidney and liver findings. Increased survival due to bw reduction, reduced food consumption, prostatitis, periodontal inflammation
Studies from the 2001 evaluation	Annex B.5.5.1 Glyphosate Monograph IIA 5.5.2/04 1993a (CHE 1)	2-year, oral diet Rat, Sprague-Dawley	0, 10, 100, 300, 1000	300 (1000)	1000 Decreased body weights, decreased urinary pH, salivary glands (histopatho-logy at terminal and interim kill, organ weight ↑ at interim kill); evidence of weak liver toxicity (clinical chemistry AP ↑, organ weight ↓)
	Annex B.5.5.1.2 Glyphosate Monograph IIA 5.5.2/05 1981 (MON 1)	26-month, oral diet Rat, Sprague-Dawley	♂ 0, 3, 10, 31 ♀ 0, 3.4, 11, 34	31/34	No treatment-related effects

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Reference (Owner**)		Type of study / Species	Dose levels (mg/kg bw/day)	NOAEL (NOAEL)* (mg/kg bw/day)	LOAEL (mg/kg bw/day) Targets / Main effects
Studies from the 2001 evaluation	Annex B.5.5.1.2 Glyphosate Monograph IIA 5.5.2/06 1990 (MON 2)	2-year, oral diet Rat, Sprague-Dawley	♂ 0, 89, 362, 940 ♀ 0, 113, 457, 1183	362/457 (940/1183)	940/1183 Systemic effects: cataracts ♂, reduced body weight in ♀, increased liver weight. Local effects: inflammation of gastric mucosa
Studies not reviewed in the 2001 evaluation	2009a (NUF 1)	2-year, oral diet Rat, Wistar	0, 95, 317, 1230	1230 (1230)	> 1230 No treatment-related effects
	IIA 5.5.3/01 , 2001 (FSG 2)	18-month, oral diet Mouse, Swiss albino	0, 15, 151, 1460	151 (1460)	1460 Increased mortality
Studies from the 2001 evaluation	Annex B.5.5.2.2 Glyphosate Monograph 1993b (CHE 2)	2-year, oral diet Mouse, CD-1	0, 100, 300, 1000	1000 (1000)	> 1000 Not clearly identified
	Annex B.5.5.2.1 Glyphosate Monograph 1983 (MON 3)	2-year, oral diet Mouse, CD-1	♂ 0, 157, 814, 4841 ♀ 0, 190, 955, 5874	157/190 (4841/5874)	841/955 Decreased body weight, histological changes in liver and urinary bladder (epithelial hyperplasia)
Studies not reviewed in the 2001 evaluation	1997 (ALS 2)	18-month, oral diet Mouse, ICR	0, 1600, 8000, 40000 ppm	8000 / 1600 ppm (= 838/153 mg/kg bw/day (♂/♀) (4348/4116 (♂/♀))	8000 ppm (≅ 787 mg/kg bw/day) (♀ only): retarded growth 40000 ppm: pale-coloured skin ♂, loose stool, retarded growth, reduced food consumption and food efficiency, caecum distension and increased absolute and relative caecum weight without histopathological findings increased incidence of anal prolapse in , consistent with histopathological erosion/ulcer of the anus
	2009b (NUF 2)	18-month, oral diet Mouse, CD-1	0, 500, 1500, 5000 ppm	810/1081 (♂/♀) 946 (♂+♀) (810/1081 (♂/♀)	No treatment-related effects

* NOAEL for carcinogenicity
** Number refers to the data presented in Figure 5.11-1.
↓ = decreased; ↑ = increased

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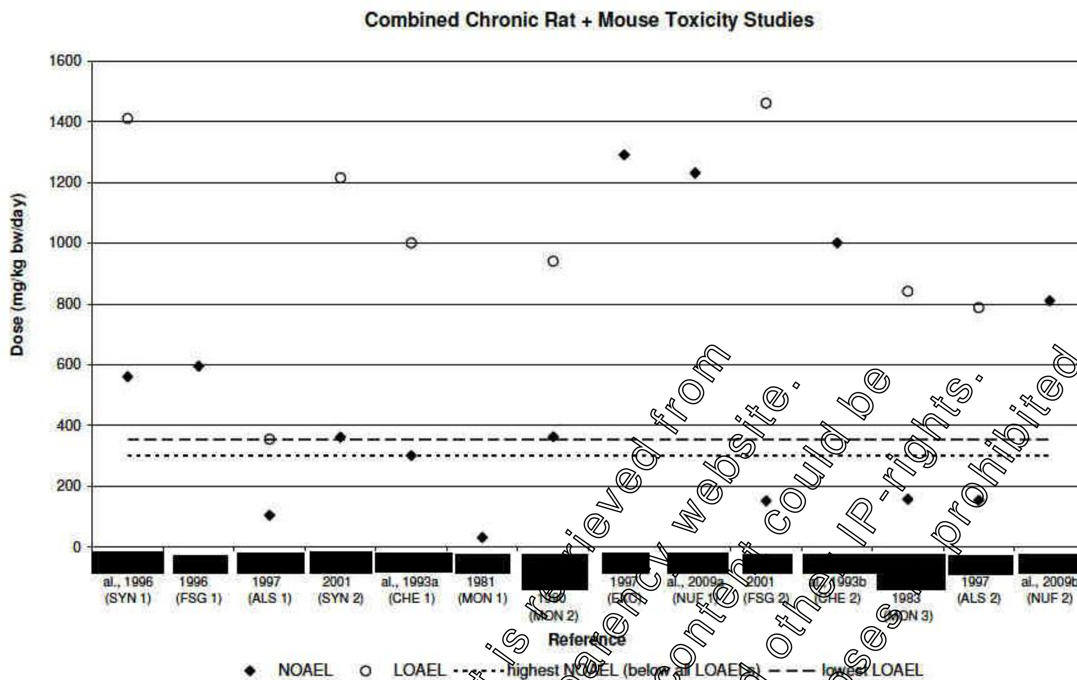
Figure 5.11-1: NOAELs and LOAELs observed in chronic rat and mouse studies with glyphosate



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The 2004 JMPR review of glyphosate established an ADI for glyphosate of 1.0 mg/kg bw/day on the basis of the NOAEL of 100 mg/kg bw/day for salivary gland alterations in a long-term study of toxicity and carcinogenicity in rats and a safety factor of 100. At that time the JMPR review of glyphosate concluded that this treatment-related effect was of unknown toxicological significance. In addition, it has to be noted that in the previous EU glyphosate evaluation the NOAEL for some 2-year rat studies were lower than presented in this review based on salivary gland effects. However, the cellular alterations observed in the salivary glands are considered to be an adaptive response to the acidic diet from glyphosate technical acid and are of no adverse consequence because:

- The effect is observed with other organic acids with a similar pH-dilution curve to glyphosate.
- The effect is only observed following treatment in the diet. The same effect has not been observed across an extensive database following other exposure routes. The ADME radiolabel studies indicate glyphosate does not accumulate in the salivary gland.
- The effect, seen primarily in the rat, is variable in severity and has not been observed consistently across sex, dose or strain.
- From a histopathological perspective across an extensive database, there is no accompanying evidence of cytotoxicity leading to necrosis or apoptosis, no evidence of inflammation or change in function and the cellular alterations do not progress with time to preneoplastic or neoplastic lesions (but in fact decrease in incidence and severity or disappear all together with time).
- The effect is reversible upon cessation of treatment with a low pH diet.

Based on sub-chronic toxicity studies performed in rats and mice, the mouse seems to be the least sensitive species. Due to the dose-spacing chosen in the long-term toxicity studies, the lowest LOAELs observed in mice were equal or greater than 841 mg/kg bw/day, with NOAELs of about 150 mg/kg bw/day and higher. The LOAELs observed in chronic rat studies were lower as compared to mice. Thus the rat is considered the most appropriate species for ADI derivation.

The highest relevant NOAEL observed in chronic toxicity studies is the **NOAEL of 300 mg/kg bw/day** from a 2-year rat study (1993). This value is supported by two other studies in rats (2001; 1990) with slightly higher NOAELs of 361 mg/kg bw/day and 362 mg/kg bw/day, respectively.

Applying a safety factor (SF) of 100 the **ADI** is considered to be **3 mg/kg bw/day** (i.e. 300 mg/kg bw/day/ 100 (SF)).

Acceptable Operator exposure Level (AOEL)

In the previous 2001 EU glyphosate evaluation the AOEL based on maternal effects observed in rabbit developmental toxicity studies. The relevant NOAEL was 75 mg/kg bw/day. In addition to the multiple rabbit developmental toxicity studies reviewed in the initial Annex I inclusion of glyphosate, three more developmental toxicity studies in rabbits (███ 1995, ███ 1996, and ███ 1996) have confirmed that adult rabbits are sensitive to oral gavage dosing with glyphosate.

In rabbits, glyphosate exposure via oral gavage led to clinical signs of toxicity consistent with gastro-intestinal disturbances. Rabbits were more sensitive to oral gavage dosing than other species. Clinical signs observed included diarrhoea/soft faeces, reduced faecal output, reduced body weights, reduced food consumption and increased mortality, all consistent with gastro-intestinal stasis (ileus). Rabbits (caecotrophs) are particularly sensitive to disruption of the gastro-intestinal tract. Stress and other environmental factors such as pain can lead to the normal muscular contractions of the stomach and intestines being greatly diminished which in turn leads to disruption of the normal intestinal/ceacum bacterial flora. It is likely that the mucosal membrane of the rabbit gastro-intestinal tract is irritated by bolus administration of glyphosate acid and that the associated stress or pain leads to gastro-intestinal stasis. The gross necropsy signs observed in maternal animals in the studies by ███ (1995), ███ (1996) and ███ (1996), such as hair-like boluses in the stomach, fluid filled large intestines and gas distension in the lower gastrointestinal tract are indicative of gastro-intestinal stasis. The severity of this finding appears to be more relevant to hindgut fermenters as both the rat and the dog appear better adapted to tolerate the irritation potential of an oral bolus dose administration of glyphosate acid.

Further evidence, that these findings are related to gastro-intestinal disturbance comes from the ███ (2012) study that measured dermal absorption *in vitro* through rabbit skin. Based on the results of this study, 2.66% of the dermally applied dose in the ███ 21-day dermal toxicity study in the rabbit (1982), where there was no evidence of gastro-intestinal tract effects, was systemically available. Thus, the NOAEL for systemic effects after dermal application of 5000 mg/kg bw in the ███ study was 133 mg/kg bw. Since lower systemic doses resulted in significant gastro-intestinal toxicity in dams from the rabbit developmental toxicity studies, such effects were likely attributable to a route specific toxicity rather than systemic toxicity.

In general the AOEL is derived from the highest dose at which no adverse effects are observed in relevant studies in the most sensitive species. Since operators are normally not exposed over long time periods to plant protection products, relevant studies for the AOEL derivation are sub-chronic (i.e. 90-day) or developmental toxicity studies.

The consistent delayed onset of symptoms in rabbit developmental toxicity studies, suggests that the effects may be due to repeated dosing of a low pH organic acid via oral gavage to the rabbit, causing local irritation of the gastric mucosa. These effects are not representative of glyphosate-related systemic toxicity, but due to gastrointestinal tract (GIT) disturbances caused by a large bolus dose of acidic material.

After dietary administration (e.g. as in the sub-chronic toxicity studies) effects observed at the LOAELs consisted mainly on systemic effects (e.g. changes in clinical chemistry parameters, decrease of urinary pH). Some effects caused by GIT disturbances (e.g. diarrhoea) were also present. However, these effects occurred at higher dose levels as compared to effects observed after gavage dosing in the rabbit.

Regarding the facts outlined above the rabbit developmental toxicity studies are considered inappropriate for derivation of the AOEL, since the observed effects are not representative of systemic toxicity.

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This is substantiated by the rationale given in the draft guidance document for AOEL derivation[11]. According to the draft guidance the dependency of the observed toxicity on the exposure route is essential for the determination of the most appropriate study for AOEL setting (see 3.14 of the guidance document).

Therefore, the sub-chronic toxicity studies performed in rodents by dietary administration of glyphosate are used for AOEL derivation, since the observed effect levels based on systemic toxicity.
The relevant NOAELs/LOAELs from 90-day toxicity studies are summarised in Table 5.11-2 and Figure 5.11-2 below.

Table 5.11-2: Relevant NOAELs and main effects for AOEL derivation

Reference (data owner)*		Type of study Species	Dose levels	NOEL / NOAEL	Targets / Main effects
Studies from the 2001 evaluation	1989 (CHE 1)	90-day, oral diet Rat, Sprague-Dawley	0, 30, 300, 1000 mg/kg bw/day	NOAEL: 300 mg/kg bw/day	1000 mg/kg bw/day: Clinical chemistry, cellular alterations in paratoid salivary glands, decreased urinary pH (♂ only)
	1993 (Alkaloida, ALK)	13-week, oral diet Rat, Sprague-Dawley	0, 2000, 6000, 20000 ppm (≅ 0, 125.2/156.3, 371.9/481.2, 1262/1686.5 mg/kg bw/day (♂/♀)	NOAEL = 6000 ppm (≅ 379.1/481.2 mg/kg bw/day (♂/♀)	1262/1686.5 mg/kg bw/day (♂/♀) (calculated values): Diarrhea, blood in urine, organ weight changes
	1992 (FSG)	90-day, oral diet (+ 2-week recovery) Rat, Wistar	0, 200, 2000, 20000 ppm	2000 ppm (147/196 mg/kg bw/day (♂/♀))	1000 mg/kg bw/day (estimated value): Clinical chemistry, reduced body weight gain
Studies from the 2001 evaluation	1989 (Barclay, BCL)	13-week, oral diet (+ 5-week recovery) Rat, CD	0, 2000, 3000, 5000, 7500 ppm	NOEL: 7500 ppm (ca. 375 mg/kg bw/day)	No treatment-related effects
	1987 (MON)	90-day, oral diet Rat, Sprague-Dawley	0, 1000, 5000, 20000 ppm	NOEL: 20000 ppm (1267/1623 mg/kg bw/day (♂/♀))	No treatment-related effects
Studies not reviewed in the 2001 evaluation	1995 (ALS 1)	13-week, oral, diet Rat, Sprague-Dawley	0, 3000, 10000, 30000 ppm (≅ 168/195, 569/637, 1262/1686.5 mg/kg bw/day (♂/♀))	NOAEL: 3000 ppm (168/195 mg/kg bw/day ♂/♀)	10000 ppm (≅ 569/637 mg/kg bw/day (1262/1686.5 mg/kg bw/day (♂/♀)): caecum distension; caecum weight increased without histopathological findings 30000 ppm (≅ 1735/1892 mg/kg bw/day (1262/1686.5 mg/kg bw/day (♂/♀)): caecum distension; caecum weight increased without histopathological findings, reduced body weight and lower food efficiency; increased AP activity in ♀
	, 1996	90-day, oral,	0. 1000, 5000,	NOEL: 5000	1612/1821 mg/kg bw/day:

[11] Working Document – Draft Guidance for the setting and application of acceptable operator exposure levels (AOELs); SANCO 7531 – rev. 10; European Commission, Health & Consumer Protection Directorate-General, 2006-07-07

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Reference (data owner)*		Type of study Species	Dose levels	NOEL / NOAEL	Targets / Main effects
	(1st revision of study) (SYN)	diet Rat, Alpk:AP_fSD	20000 ppm	ppm (414/447 mg/kg bw/day ♂/♀	Reduced body weight, food consumption and utilisation reduced in ♂ only, clinical chemistry changes (↑ ALP, ALT)
	1996 (NUF)	90-day, oral, diet Rat, Sprague-Dawley	0, 1000, 10000, 50000 ppm (≅ 0, 79/90, 730/844, 3706/4188 mg/kg bw/day (♂/♀))	1000 ppm (79/90 mg/kg bw/day ♂/♀)	730/884 mg/kg bw/day: Clinical chemistry changes, mucosal atrophy of the caecum
	1995 (ALS 2)	13-week, oral, diet Mouse, ICR	0, 5000, 10000, 50000 ppm (≅ 0, 600/765, 1221/1486, 6295/7435 mg/kg bw/day (♂/♀))	NOAEL: 5000 ppm (600/765 mg/kg bw/day ♂/♀)	10000 ppm (1221/1486 mg/kg bw/day ♂/♀): caecum distension ♀; increased absolute and relative caecum weight 50000 ppm 6295 / 7435 mg/kg bw/day ♂/♀):: reduced bodyweight and food consumption, decreased food efficiency ♀, haematological changes in ♀, blood chemistry changes, caecum distension and increased absolute and relative caecum weight in both sexes without histopathological changes in the caecum; cystitis of the urinary bladder in ♂
Studies from the 2001 evaluation	1991 (CHE 2)	13-week, oral, diet, Mouse, CD-1	0, 200, 1000, 4500 mg/kg bw/day	NOEL: 4500 mg/kg bw/d	No treatment-related effects

*: Number refers to data presented in Figure 5.11-2.
↓ = decreased; ↑ = increased;

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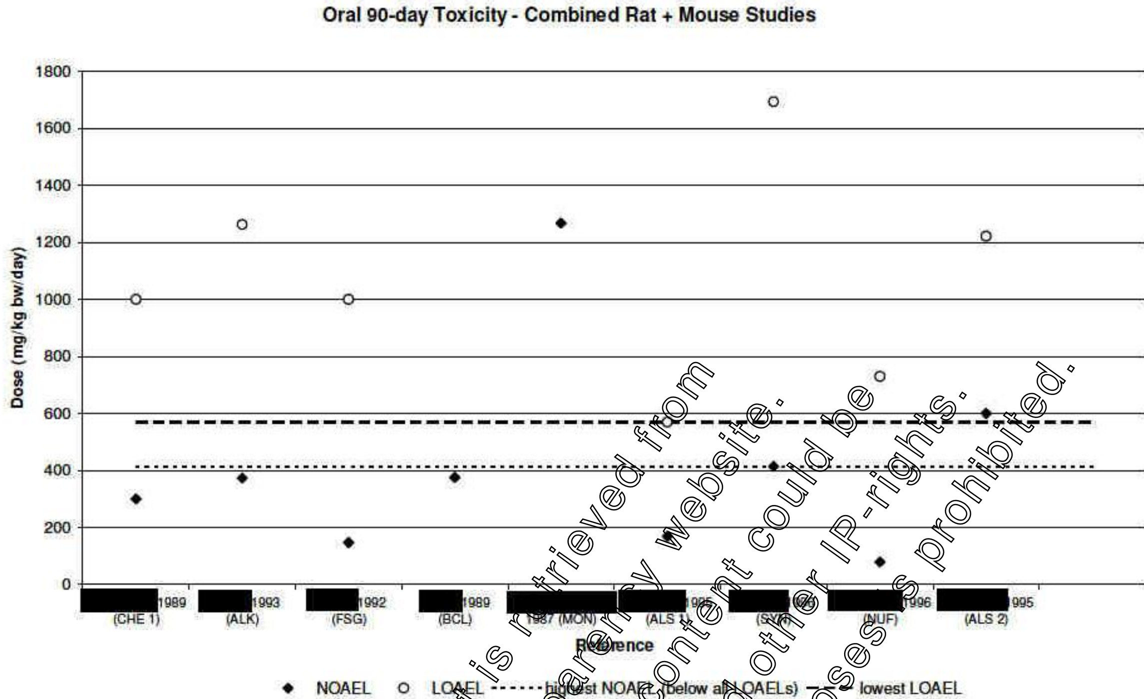
Figure 5.11-2: NOAEL / LOAELs from 90-day toxicity studies in rodents



*The NOAEL of 4500 mg/kg bw/day derived from from ██████ 1991 (CHE 2) was not considered in this Figure

As can be seen from the graph and table above, the 90-day rat study conducted by ██████ 1996 (SYN) provides the most sensitive endpoint. The observed **NOAEL was 414 mg/kg bw/day**.

With a safety factor of 100, as well as a correction for 30% oral absorption, the resulting AOEL **is 1.2 mg/kg bw/day**.

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Maximum Acceptable Concentration in drinking water (MAC_{DW})

Glyphosate is an organic herbicide and is thus a pesticide as defined by the Drinking Water Directive 98/83/EC. Thus, based on the arbitrarily mandated non-risk based value for single pesticides, the **limit concentration** of glyphosate in drinking water is considered **0.1 µg/L**.

In addition to the regulatory defined limit concentration, the drinking water limit can be scientifically derived using the ADI as starting point. Allowing 10 % of the ADI to be contributed by drinking water and assuming that an adult person of 60 kg bodyweight consumes 2 L water per day, the **MAC_{DW}** is calculated to be **9 mg/L** (i.e. MAC_{DW} = (ADI x 0.1 x 60 kg bw/2 L)).