Potential toxic effects of glyphosate and its commercial formulations below regulatory limits

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Abstract

Glyphosate-based herbicides (GlyBH), including Roundup, are the most widely used pesticides worldwide. Their uses have increased exponentially since their introduction on the market. Residue levels in food or water, as well as human exposures, are escalating. We have reviewed the toxic effects of GlyBH measured below regulatory limits by evaluating the published literature and regulatory reports. We reveal a coherent body of evidence indicating that GlyBH could be toxic below the regulatory lowest observed adverse effect level for chronic toxic effects. It includes teratogenic, tumorigenic and hepatorenal effects. They could be explained by endocrine disruption and oxidative stress, causing metabolic alterations, depending on dose and exposure time. Some effects were detected in the range of the recommended acceptable daily intake. Toxic effects of commercial formulations can also be explained by GlyBH adjuvants, which have their own toxicity, but also enhance glyphosate toxicity. These challenge the assumption of safety of GlyBH at the levels at which they contaminate food and the environment, albeit these levels may fall below regulatory thresholds. Neurodevelopmental, reproductive, and transgenerational effects of GlyBH must be revisited, since a growing body of knowledge suggests the predominance of endocrine disrupting mechanisms caused by environmentally relevant levels of exposure.

Keywords: glyphosate, Roundup, GMO, endocrine disruption, toxicity, pesticide

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1. Background, aim and scope

Glyphosate-based herbicides (GlyBH), mainly represented by Roundup, are the most widely used commercial formulations of pesticides worldwide (European Commission, 2007; US EPA, 2012). Glyphosate is the active ingredient of more than 750 different broad-spectrum herbicides (Guyton et al., 2015). GlyBH are used on food and feed crops during cultivation, to desiccate the crop before harvest (for instance, wheat), and more intensively during the cultivation of the 80% of genetically modified (GM) plants that are engineered to tolerate GlyBH (James, 2014). Glyphosate represented 3.7% of the mass of total herbicide active ingredient applied in 1996 in the US, but 53.5% in 2009 (Coupe and Capel, 2015).

Glyphosate acts on the shikimate pathway in plants through the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Boocock and Coggins, 1983), which is involved in the metabolism of aromatic amino acids. Inhibition of EPSPS by glyphosate causes protein shortage, and consequently plant death. Since this biochemical pathway does not exist in vertebrates, it is generally assumed that glyphosate is safe for mammals, including humans (Williams et al., 2012). As a consequence, glyphosate jumped to a leading position among commercial pesticides from the 1970s. GlyBH use is still increasing every year (Benbrook, 2012). This use is driven predominantly by adoption of agricultural GM plants that have been designed to tolerate the Roundup herbicide, and require more and more sprays due to weed resistance. While the use of GlyBH is driven by their association with Roundup-tolerant GMOs, GlyBH residues can also be found in so-called “GMO-free” food and feed because these herbicides are increasingly used for pre-harvest crop desiccation.

GlyBH-tolerant GM plants do not metabolize or excrete glyphosate, and therefore accumulate it during their growth (Arregui et al., 2004). While other pesticides are generally allowed in edible plants at levels around 0.01-0.1 ppb (DG SANCO, 2013), glyphosate and its metabolite aminomethylphosphonic acid (AMPA) have among the highest maximum residue limits (MRL), with up to 500 ppm (calculated as the sum of glyphosate + AMPA) authorized in some feed. The MRL in transgenic soybean, a major edible GMO grown for livestock feed, has been set at 20 ppm. In 2011, the U.S. Department of Agriculture reported residues of glyphosate and AMPA in 90.3% and 95.7% soybean samples, respectively at the levels of 1.9 ppm and 2.3 ppm. An MRL of 2 ppm has been set for bovine kidney, since cattle are increasingly fed transgenic Roundup Ready GM soy. Indeed, farm animal feeding studies showed levels of glyphosate in kidney and liver that are around 100 fold greater than

the levels found in fat or muscles (Germany Rapporteur Member State, 2015). Residues of glyphosate and AMPA have also been found to contaminate surface waters, even in areas without GMO crops (Coupe et al., 2012; IFEN, 2006). The ubiquity of glyphosate in food/water means that it is regularly ingested. The real contamination of populations by Roundup residues is poorly characterized. The US Centers for Disease Control and Prevention provide an extensive survey of population's exposure to 250 commonly used industrial chemicals, but these do not include glyphosate. Based on limited studies using small cohorts, it is estimated that glyphosate is regularly found in urine at levels corresponding to a dietary daily intake of around 0.1-3.3 µg/kg bw/d (Niemann et al., 2015).

Reviews of GlyBH health effects have been performed by governmental agencies (EPA, 1993; European Commission, 2002), by scientists on behalf of companies selling GlyBH (Greim et al., 2015; Mink et al., 2011; Williams et al., 2012; Williams et al., 2000), or by independent academics (Antoniou, 2012; Astiz, 2009; López et al., 2012; Székács, 2012). All these reviews report conflicting opinions, especially for long-term effects of glyphosate and its commercial formulations. In Europe, the new glyphosate threshold for long-term toxicity (established on rats) is 350 mg/kg bw/d, based on liver dysfunctions (Germany Rapporteur Member State, 2015). The no-observed-adverse-effect level (NOAEL) was 100 mg/kg bw/d. The new proposed Acceptable Daily Intake (ADI) was calculated from the lowest NOAEL in rabbit developmental studies (50 mg/kg bw/d). Taking into account a safety factor of 100 (10 for intraspecies and 10 for interspecies variabilities), ADI has been calculated at 0.5 mg/kg bw/d. From the same data, the USA equivalent of the ADI, the reference dose (RfD), was calculated at 1.75 mg/kg bw/d (EPA., 2009). In this case, the LOAEL was considered to be 350 mg/kg bw/d (the NOAEL being 175 mg/kg bw/d) from rabbit teratogenicity studies. It should be emphasized that doses used in regulatory toxicity experiments, generally ranging from 10 to 1000 mg/kg/d, are not representative of human environmental exposures, which occur at the level of µg/kg bw/d (Niemann et al., 2015).

We performed a review of effects of glyphosate and its formulations on laboratory mammals below these regulatory limits, taking into consideration all data relative to mammalian glyphosate and GlyBH toxicities. A literature review was performed on Science Direct and PubMed databases using the keywords "glyphosate", "N-(phosphonomethyl)glycine" and "Roundup" (until April 2015). We also used our personal bibliography database generated by a 10-year scientific literature follow-up.

We did not report short-term studies or studies with doses resulting in acute effects, in other words with doses above the regulatory threshold for long-term toxicity (350 mg/kg bw/d), because they are not a matter of debate. Indeed, ADI or RfD is clearly exceeded in some accidental and intentional exposures. This is often through handling accidents or suicide attempts by farmers. These generally one-off exposures are in the range of acute intoxication doses. The most common symptom recorded after 4000 GlyBH accidental exposures is a mild transient gastrointestinal impairment (Roberts et al., 2010). GlyBH also affect the cardiovascular system at acute doses (Gress et al., 2014), the underlying electrophysiological mechanisms have been studied (Gress et al., 2015). Death was strongly associated with greater age, larger ingestions and high plasma glyphosate concentrations on admission (>734 µg/mL) (Roberts et al., 2010). Extreme exposure (around 100-200 mL of the pure formulation ingested) resulted in respiratory, heart and hepatorenal damage (Bradberry et al., 2004). In intentional ingestions (suicide attempts), up to 500 mL are ingested (Potrebic et al., 2009).

In order to include some results of regulatory tests on glyphosate alone, we used regulatory reports that served as a basis for glyphosate commercial authorization in Europe and USA. However, we were limited by the unpublished status and confidentiality of the pre-commercialization tests included in these reports. We asked the French agency for food, environmental, and occupational health and safety (ANSES) for the raw data for the health assessment of GlyBH and glyphosate. ANSES was not in possession of all the raw data on glyphosate. Also, data on the short and long-term effects of Roundup consumption on blood parameters were lacking (Mortureux, 2013). For Europe, we used the German authorities' draft assessment report (DAR) on the industry studies (Germany Rapporteur Member State, 2015). Germany is the rapporteur for the market release of glyphosate in the European Union. As studies and raw data summarized in the DAR were not publicly available, we were not able to independently assess the studies; thus we have considered summary data. Health evaluation in the DAR was mostly based on studies provided by the Glyphosate Task Force (25 companies joining resources in order to renew the European glyphosate registration). Some were amended by deletion of redundant parts and corrections of obvious errors (Germany Rapporteur Member State, 2015). Each new study was commented. Studies that were part of the previous EU evaluation were also subjected to reassessment according to current quality standards. A wide range of technical databases have been used for the literature search to create the DAR. This is thus the most comprehensive regulatory report, grouping results from 150 new toxicological studies and considering results of 900 publications from scientific journals, among which 200 publications were reviewed in detail.

For the USA, we used the 2011 US Forest Service risk assessment on glyphosate (USDA Forest Service, 2011) and the US EPA 1993 Reregistration Eligibility Decision (RED) Fact Sheet (U.S.EPA, 1993).

2. Other ingredients added in commercial formulations

GlyBH formulations are generally made of around 36-48% glyphosate, water, salts, and adjuvants such as ethoxylated alkylamines (POEA). Glyphosate is never used without its adjuvants, which allow and enhance its herbicidal activity by promoting its toxicity. Adjuvants are however considered and declared as inert diluents because they are not considered to be directly responsible for the pesticide activity. They are classified as confidential for regulatory purposes. However, the fact that an ingredient of a mixture (glyphosate in the formulation) is active in plants does not mean a priori that this ingredient is the most toxic of the mixture, neither for humans or other levels of biodiversity. There is an unexpressed, widely believed assumption that the active principle against plant metabolism (glyphosate) is the most toxic compound of GlyBH formulations on non-target species. At a regulatory level, glyphosate is tested alone on mammalian health in long-term in vivo chronic, developmental and reproductive studies. It leads to ADI calculations and other regulatory norms for glyphosate alone, even though it is never used in this form but only as part of a mixture with adjuvants in the commercial formulations.

Formulations vary between different brands and between different countries. As a result of this variability in adjuvants, and since most of them are not compulsorily declared, GlyBH effects are complex and the result of mixture effects. Consequently, the described effects in literature vary widely. In fact, not all authors clearly indicate which GlyBH they have used (Chan et al., 2007; Hokanson et al., 2007; Sivikova and Dianovsky, 2006), confusing the products or the adjuvants (Contardo-Jara et al., 2009; Gehin et al., 2005). For instance, not all adjuvant mixtures contain POEA. “Glyphosate” is often written for “Roundup” (Cavusoglu et al., 2011; George et al., 2010), as visible in the materials and methods, or “Roundup (glyphosate)” is written as if Roundup were equivalent to glyphosate alone (Stachowski-Haberkorn et al., 2008). Thus, it is not even clear if the authors are assessing glyphosate or its formulations.

3. Hepatic and kidney toxicity

The liver and kidneys are among the first endpoints of alimentary intoxications. Because of observed increases in the frequency of chronic kidney disease among farmers (Jayasumana et al., 2015), possible kidney and liver effects of GlyBH exposures is a matter of concern.

Summary of regulatory toxicity studies

Chronic hepatorenal toxicities were investigated for glyphosate by the manufacturer in combined chronic toxicity/carcinogenicity studies based on Organization for Economic Cooperation and Development (OECD) 453 guideline. We have not analyzed acute studies (28 days or less) because they generally use dosages in the range of lethal doses, or shorter to show undiscussed irritating properties, without blood analyses. These are not very informative for the side effects in cases of environmental exposures. The acute exposure symptoms have features and underlying mechanisms already well known (Bradberry et al., 2004; Roberts et al., 2010).

In the last glyphosate regulatory assessment performed for the European authorization, in long-term studies in rats, 100 mg/kg bw/d is considered to represent an overall NOAEL based on the combined assessment of four studies (Germany Rapporteur Member State, 2015). The overall LOAEL was considered to be 350 mg/kg bw/d, even if alterations in clinical chemistry parameters were observed at lower doses. We have summarized the long-term toxicity studies present in the last European rapporteur report (Germany Rapporteur Member State, 2015).

In one glyphosate (alone) study (report reference IIA, 5.5.2/04), differences in the serum and urine biochemistry were noticed and reported. Among them, alkaline phosphatase (AP) activity was increased from 10 mg/kg bw/d, in both sexes at 5 sampling times, although the difference was not always statistically significant. At the interim sacrifice, absolute liver weights were reduced in males and females at doses of 100 mg/kg bw/d and above. Another histopathological finding at the same doses was a decreased incidence of nephropathy in treated males. Two other studies with doses of ~3, 10 and 30 mg/kg bw/d for reference IIA, 5.5.2/05 study, and of ~100 mg/kg bw/d for reference IIA, 5.5.2/06 study, resulted in some occasional biochemical changes which were not considered to be treatment-related and were thus not reported in the assessment report. The fourth study

(reference IIA, 5.5.2/01, doses of 7.4, 73.9, and 740 mg/kg bw/d), resulted in toxic effects in liver at the highest level.

For the new 2014 evaluation, 5 long-term studies of glyphosate in rat were added (Germany Rapporteur Member State, 2015). The first study (IIA, 5.5.1/01), performed by Syngenta, used Wistar rats treated with doses from 154 mg/kg bw/d and found a treatment- and dose-related increase in AP activity. The second study (IIA, 5.5.2/02) reported general toxicological effects at high levels consistent with symptoms of acute intoxication. In the low dose group (around 100 mg/kg bw/d), there was a significant decreased spontaneous motor activity and a significant increased bradypnea and soiled fur in males. At the same dose and above, there was also an increased AP activity. Females in this group presented a significant increase of the incidence of ptosis and tactile hair loss. The third study (IIA, 5.5.2/07) used high levels that were not consistent with our inclusion criteria descrided above. In the fourth study (IIA, 5.5.2/03), with glyphosate doses of 133, 399 and 1356 mg/kg bw/d, a trend toward an increased AP activity (not always statistically significant) was observed from the low dose. Other changes observed at the two highest levels are not detailed here. For the fifth study (IIA, 5.5.2/08), Wistar rats were treated with 95, 317 and 1230 mg/kg bw/d. A transient increase in AP activity was observed, confirming findings in many other studies with glyphosate.

The first mice study (IIA, 5.5.3; dose levels of 15, 151, and 1460 mg/kg bw/d), the second mice study (IIA, 5.5.3/02; dose levels of 85, 267, and 946 mg/kg bw/d), and the third long-term study in mice (IIA 5.5.3/03; doses levels of 159, 812 and 4232 mg/kg bw/d) did not report toxic effects in liver or kidneys at relevant levels. Details on other mice toxicity studies were not included in the European rapporteur report (Germany Rapporteur Member State, 2015).

Peer-reviewed literature

Mechanisms of glyphosate toxicity in liver are well understood. Hepatic effects of glyphosate have been known since the 1980s, among them the ability of glyphosate to disrupt liver mitochondrial oxidative phosphorylation from 15 mg/kg bw in rats (Olorunsogo et al., 1979). A decrease in succinate-dependent respiratory indexes of rat liver mitochondria is observed with 85 mg/L of glyphosate in Roundup (Peixoto, 2005). In fact, glyphosate interacts at the succinate binding site of mitochondrial succinate dehydrogenase (Ugarte, 2014). An electron microscopy analysis after Roundup hepatocyte exposure has shown a

reduced respiratory activity and a decreased transcriptional/splicing activity (Malatesta et al., 2008).

Glyphosate is also a strong chelator of metal cations such as copper, manganese, cobalt, iron, and zinc, as well as calcium and magnesium (Lundager Madsen et al., 1978), and was initially patented for this feature (U.S. Patent No. 3,160,632, 1964). Glyphosate's ability to act as a chelator also partly explains its uncoupling effects on the mitochondrial chain (Olorunsogo, 1990). Additionally, the fact that glyphosate acts as a protonophore (Olorunsogo, 1990), increasing mitochondrial membrane permeability to protons and Ca^{2+}, can also explain oxidative stress induced by glyphosate alone (Astiz et al., 2009a) or its formulations in vivo (El-Shenawy, 2009), with molecular understanding from in vitro experiments (Gehin et al., 2006). Indeed, Ca^{2+} is considered to be one of the major stimulator of mitochondrial reactive oxygen species (ROS) accumulation because it promotes structural alterations of the inner mitochondrial membrane (Kowaltowski and Vercesi, 1999). It can also alter the mitochondrial respiratory chain, since most components of this system are integral inner mitochondrial membrane proteins (Kowaltowski and Vercesi, 1999).

These data stand in addition to the chelating or vesicle-forming capacities of adjuvants, as part of their intrinsic detergent properties. Indeed, Roundup formulations have been demonstrated to be more toxic than glyphosate alone at the mitochondrial level because adjuvants can induce a non-specific membrane permeabilization in rat isolated mitochondria (Peixoto, 2005). This oxidative stress also explains damage in other organs. A 30 minutes Roundup exposure of rat primary testicular cell at 36 ppm induces oxidative stress and activates multiple stress-response pathways leading to Sertoli cell death in prepubertal rat testis (de Liz Oliveira Cavalli et al., 2013). Glyphosate at a dose of 10 mg/kg bw/d, administered 3 times a week, stimulated the antioxidant defense system in the liver, kidney, brain and plasma of rats. (Astiz et al., 2009c).

Nevertheless, most results were obtained at doses greater than human population exposures. We have performed a 2-year-long study using groups of 10 Sprague-Dawley rats, which were administered with 0.1 ppb of a Roundup formulation (containing 45 ng/L of glyphosate mixed with adjuvants) in drinking water (Séralini et al., 2014). This level corresponds to an admissible concentration of GlyBH residues in drinking water. We revealed signs of hepatorenal toxicities, as well as urine and blood biochemistry and hormonal disturbances at the 15th month. In another study, glyphosate administered to rats

at a concentration of 4.87 mg/kg bw glyphosate every 2 days over 75 days induced hepatic leakage of ALAT and ASAT, suggesting irreversible damage in hepatocytes (Benedetti et al., 2004). Glyphosate exposure of rats at 0.09 mg/kg bw/day (0.7 ppm in drinking water), a level allowed in Brazilian inland waters (US EPA, 2011), caused an increased in glutathione levels and enhanced glutathione peroxidase activity in liver and kidneys (Larsen et al., 2012). As a consequence of liver intoxication, Roundup triggers the activation of xenobiotic-metabolizing enzymes. CYP1A1/2 and CYP3A dependent enzymes were inhibited in male rats from 0.7 ppm dissolved in water after a 90-day exposure (Larsen et al., 2014). Disruptions of CYP1A1/2 and CYP3A enzymes were observed at levels as low as 0.1 ppb in drinking water for a life-long exposure (Séralini et al., 2014), and corroborated in hepatocyte cell lines (Gasnier et al., 2011).

Commentary on liver and kidney toxicity

There is a coherent body of evidence showing that glyphosate and its commercial formulations can cause oxidative stress, leading to organ damage. Oxidative stress occurs because of an imbalance between factors creating a pro-oxidative environment and cellular antioxidant defense system (Zhu et al., 2012). Reactive oxygen species are highly reactive molecules and could damage cellular molecules such as lipids, proteins or DNA. Excessive oxygen free radicals lead to cell damage (Zhu et al., 2012). Such damage can be reflected by increased alkaline phosphatase (AP) or aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) activities (Yamada et al., 2006).

While the new regulatory assessment of glyphosate's toxic effects established the NOAEL and the LOAEL for chronic toxic effects at respectively 100 and 350 mg/kg bw/d, the previous assessment by the same agency (German Federal Agency CPFS, 1998) considered the NOAEL and the LOAEL to be respectively 30 and 60 mg/kg bw/d. They were used to calculate an ADI at 0.3 mg/kg bw/d. Indeed, there is a coherent pattern showing toxic effects on the liver (marked by an increased AP activity) across all studies. A trend can be observed from 10 mg/kg bw/d, the statistical significance being reached at various doses depending on the studies. Indeed, results across the different regulatory studies are highly heterogeneous. Some studies detected signs of acute intoxication from 100 mg/kg bw/d while some did not report adverse effects at much higher doses. These differences could arise from differential contaminations of the tested substance. Indeed, contaminants were detected in a filtrate of glyphosate performed by Monsanto (Smith and Barclay, 1992), such as phosphonomethyliminodiacetic acid (100 ppm), N-formyl glyphosate (100 ppm), hydroxymethylphosphonic acid (20 ppm), phosphate (200 ppm), phosphite (40 ppm), imido-

bis-methylene phosphonic acid (350 ppm), methylglyphosate (600 ppm), formic acid (1.50%), AMPA (3500 ppm), and formaldehyde (2.85%). Moreover, rodent laboratory feed is also a source of contamination. The presence of feed contaminants at toxic levels could mask the toxic effects of tested substances (Mesnage, Defarge, et al., 2015). Indeed, a background rate of pathologies due to potential feed contaminants necessitates the use of high numbers of animals in order to detect statistical differences in chronic toxicity tests, because some animals die due to chronic diseases before the end of the test. Moreover, most of the statistical differences obtained in the presented regulatory tests were dismissed because they were in the range of historical data, or were not significant for all doses or all sampling times. Historical data as references are questionable, since animals have received food and water contaminated by pesticides, among other possible uncontrolled variables (Mesnage, Defarge, et al., 2015).

Toxic effects are reported at lower levels in the peer-reviewed literature, from 0.1 ppb of the commercial formulation diluted in water for long-term effects, and 0.09 mg/kg bw/d for glyphosate alone after a subchronic exposure. In general, few studies have investigated chronic toxic effects at environmentally relevant levels. Liver effects in laboratory animals and their mechanisms in cell culture model systems are corroborated by a farm study in which markers of liver dysfunction measured in cows (glutamate dehydrogenase, glutamate oxaloacetate transaminase and creatinine kinase) were statistically correlated with their urinary glyphosate levels (Krüger et al., 2013). Overall, in the peer-reviewed literature, at least 12 studies reported toxic effects of glyphosate or its commercial formulations at doses below the regulatory LOAEL for chronic toxic effects (Table 1). Most of these studies were not chronic studies and were of short duration. Three studies even reported hepatorenal changes below the ADI at levels relevant for environmental exposures (Larsen et al., 2014; Larsen et al., 2012; Seralini et al., 2014a). However, their pharmacotoxicological relevance needs to be ascertained by replications.

4. Neurotoxicity

Pesticides in general, as other pollutants such as plasticizers, are stable disruptors of cell-cell communications (Mesnage and Séralini, 2014). Thus xenobiotics not only disrupt the endocrine system but also interact with nervous system functions (Burns et al., 2013). This is typically the case for organophosphates that inhibit acetylcholinesterase activity at the

neuromuscular junction. This endpoint has been a matter of debate for glyphosate. One study reported glyphosate effects on serum acetylcholinesterase in vitro (El-Demerdash et al., 2001). However, the IC50 (half maximal inhibitory concentration) appears to be very high (714.3 mM). Even if glyphosate is structurally related to an organophosphate, the lack of a specific chemical group indicative of neurotoxicity (such as a halide, sulfur, or thiocyanate group on the phosphorus atom of glyphosate) was considered by the US EPA to be a sufficient reason to avoid the neurotoxicological assessment of glyphosate or GlyBH (the acute and 90-day neurotoxicity screening battery in the rat) (EPA, 1993). In 2009, EPA published a registration review noting that data regarding the effects of glyphosate on neurological and immune parameters are limited (EPA, 2009). Therefore, the agency anticipates requiring acute and subchronic neurotoxicity studes as well as an immunotoxicity study on glyphosate. The U.S. EPA's final re-registration decision on GlyBHs is scheduled for completion in 2015.

While the neurotoxic effects of glyphosate and GlyBH remain uncertain, especially at environmentally relevant doses, several studies in non-mammalian species have shown an inhibition of acetylcholinesterase by glyphosate. For instance, glyphosate inhibited acetylcholinesterase in the brain of *Cnesterodon decemmaculatus* from 1 ppm (Menendez-Helman et al., 2012). This enzyme is in fact inhibited in various models by doses of GlyBH in the order of ppm in *Cyprinus carpio* (Cattaneo et al., 2011), *Prochilodus lineatus* (Modesto and Martinez, 2010), amphibian tadpoles (Lajmanovich et al., 2011), *Leporinus obtusidens* (Glusczak et al., 2006; Salbego et al., 2010), and silver catfish (Glusczak et al., 2007).

Glyphosate is a derivative of glycine. As a consequence, glyphosate could inhibit serine hydroxymethyltransferases enzyme activities, a major source of intracellular glycine. Glycine consumption is a hallmark of rapidly proliferating cell (Jaim et al., 2012). Antagonizing glycine uptake and biosynthesis preferentially impaired rapidly proliferating cells. For these reasons, glyphosate has laso been suggested to inhibit cellular proliferation through depleting glycine (Li et al., 2013). The fact that glycine and other amino acids like glutamate act as neurotransmitters and play an important role in brain function raises the question of the potential neurological effects of glyphosate. The potential of glyphosate to act as a neurotransmitter is supported by its structural similarity to the glutamate receptor agonist 2-amino-3-phosphonopropionic acid. GlyBH exposure induces glutamate excitotoxicity through L-VDCC and NMDA receptor activation in immature rat hippocampus, by reducing glutamate uptake and metabolism within glial cells, and by increasing glutamate release in the synaptic cleft (Cattani et al., 2014).

The effects of oxidative stress at relatively high levels were demonstrated on brain function. The brains of glyphosate-treated rats at 10 mg/kg bw showed an increased lipid peroxidation, protein carbonylation and nitrite formation, with a decrease of alpha-tocopherol (Astiz et al., 2009c). In another study at the same doses, the same group showed a loss of mitochondrial transmembrane potential and cardiolipin content in the substantia nigra of the brain due to glyphosate (Astiz et al., 2009b). They also confirmed an increase in fatty acid peroxidation.

In epidemiology, an increase in ADD (Attention Deficit Disorder)/ ADHD (Attention deficit hyperactivity disorder) was reported in children of Minnesota farmers applying GlyBH (OR = 3.6; 95% CI: 1.35–9.65) (Garry et al., 2002). Monsanto-supported authors answered that the diagnosis had not been confirmed by a clinician (Mink et al., 2011). However, the lead author of the original study does not agree with the Monsanto authors' interpretation. We contacted Dr. Garry, who commented, "That is misleading. The diagnosis of ADHD was usually made by a psychologist, with or without referral from paediatrician (clinician) or general practitioner (clinician). In other cases the diagnosis was made by a paediatrician" (personal communication). Neural tube defects were also associated with mothers' exposures to GlyBH in a single pesticide model (OR = 1.5; 95% CI: 1.0–2.4) (Rull et al., 2006). Monsanto-supported authors dismissed this study because mothers were only considered 'exposed' if the pesticide was sprayed within 1 km of their residences, and because none of the low bound 95% confidence intervals were superior to 1.0 (Mink et al., 2011).

5. Tumorigenicity and carcinogenicity

Cancer initiation, promotion and development is a long-term process, involving multiple metabolic pathways, that can last several years, during which some tumors can become cancers. The study of this long process necessitates considerable research funding; this is usually more possible for a company than for a public research laboratory. For these reasons, only a few carcinogenic or long-term toxicity studies have been performed independently of the companies for any chemical.

Carcinogenicity of glyphosate is a complex and controversial issue. In order to support glyphosate re-approval, several reviews have been published by paid consultants of Monsanto Company (Kier, 2015; Kier and Kirkland, 2013; Mink et al., 2012) or by the glyphosate task force (Greim et al., 2015). Fourteen carcinogenicity studies (nine rat and five mouse) were evaluated for the recent European assessment of glyphosate. In contrast with raw data on chronic toxic effects, which is still kept secret, raw data on carcinogenicity studies have been published. These studies were performed according to OECD guidelines requirements with doses ranging from 3 to ~5000 mg/kg bw/day. Experiments started on young adults animals (age of several weeks) and were terminated at 2 years, before rodent aging. Some of these experiments were considered reliable even though half of the animals of some groups were not analyzed – without justification. Overall, the authors concluded that there was no evidence of a carcinogenic effect related to glyphosate treatment (Greim et al., 2015). In contrast, using partly the same data, the International Agency for Research on Cancer (IARC) has classified glyphosate as a probable human carcinogen (2A) (Guyton et al., 2015). The mechanistic evidence (genotoxicity and oxidative stress) provided independent support of the 2A classification (probably carcinogenic to humans) based on evidence of carcinogenicity in humans and experimental animals. Four key epidemiology studies of non-Hodgkin leukemia incidences were considered by the IARC panel. The 3 case-control studies, adjusted for other pesticides, indicated a statistically significant positive association. The last study, the agricultural health cohort study, did not bring additional support for association, but did not contradict the other studies. The IARC panel also noticed that glyphosate induced a positive trend for the incidence of renal tubule carcinoma and haemangiosarcoma in male mice (Guyton et al., 2015). Glyphosate also increased pancreatic islet-cell adenoma in male rats in two studies. Evidence of carcinogenicity in humans was further supported by a meta-analysis of occupational exposure to agricultural pesticides, which showed a positive association between glyphosate exposure and non-Hodgkin lymphoma subtypes (Schinasi and Leon, 2014).

In fact, glyphosate may also act as a tumor promotor, due to potential metabolism and endocrine disrupting effects. Cancer can arise from non-genotoxic compounds (Fielden et al., 2007) and tumors are frequently observed after exposure to endocrine disruptors which are not known to be genotoxic (Acevedo et al., 2013; Nishiyama et al., 2006), but which may have epigenetic effects, for instance. Tumor-promoting potential of a GlyBH on 7,12-Dimethylbenz[a]anthracene (DMBA) tumor induction was observed in mice exposed to a GlyBH at 25 mg/kg bw on the skin over a 130-day period, with a total observation of 32 weeks (George et al., 2010). A single topical application of DMBA was done, followed 1

week later by topical treatment of GlyBH, 25 mg/kg bw 3 times per week. In total, 9 markers indicating skin carcinogenesis were disrupted. At the end, 40% of animals had tumors in the DMBA + GlyBH group. Tumors were not observed with DMBA or GlyBHs alone.

We have tested (Séralini et al., 2014a) the whole Roundup pesticide formulation in rats at environmentally relevant exposure levels from 0.1 ppb in water. Our results suggested a tumorigenic non-linear and hormone-dependent effect of very low doses of Roundup (0.1 ppb) on mammary glands (2.5-fold statistically significant increase in treated females). An in vitro study has reported that glyphosate can promote the growth of estrogen-dependent human mammary breast cancer cells from 0.1 ppt through estrogenic mechanisms (Thongprakaisang et al., 2013). In this study, the transcription of estrogen responsive elements was increased by 5-13 times in presence of glyphosate. While this information can only be useful in hazard identification, concentrations used are in the range of environmental exposures. However, the reproducibility and the relevance for in vivo situations need to be confirmed by other studies. Alterations of estrogenic gene expression have also been demonstrated in another study investigating transcriptome responses of mammary cells to a GlyBH at higher levels (Hokanson et al., 2007). In another study, glyphosate and Roundup induced similar proliferative effects on MCF-7 mammary cells in both normal and charcoal-dextran-treated fetal bovine serum, suggesting a non-estrogenic mechanism of proliferation (Lin and Garry, 2000).

In the only peer-reviewed long-term study of glyphosate carcinogenic effects published in the literature, groups of 85 Wistar-RIZ outbred herd rats were exposed to 300, 900 or 2700 ppm glyphosate in drinking water. No differential tumor incidence was noticed. Only 3 fibroadenomas were developed out of 85 female controls. Indeed, some Wistar strains are particularly insensitive to carcinogens, even to strong tumor initiators like 7,12-dimethylbenz[α]anthracen (DMBA) and N-methyl-N-nitrosourea (Ahlers et al., 1998).

Even if examples of carcinogenicity not associated with genotoxicity are well known (Hayashi, 1992), the central dogma in carcinogenesis generally implies that genotoxic effects underlie carcinogenesis even if this idea is evolving when signaling pathways are chronically disrupted in pre-carcinogenic cells. The literature for Roundup genotoxicity and mutagenicity is quite extensive but also very controversial. DNA damage following Roundup or glyphosate exposure at high levels has been found in various species, such as rats, tadpole, bovine, drosophila, goldfish, caiman, eel, and humans (Bolognesi et al., 1997; Cavas and Konen, 2007; Clements et al., 1997; Gasnier et al., 2009; Guilherme et al., 2010;

Kaya et al., 2000; Lioi et al., 1998; Peluso et al., 1998; Poletta et al., 2009). In the last review (Kier and Kirkland, 2013) performed by a private consultant and former employee of the Monsanto company, on behalf of the of the glyphosate task force, 66 in vitro and in vivo genotoxicity assays were reviewed. Most of the assays performed with glyphosate alone were negative, indicating that glyphosate does not have a direct DNA-reactive mechanism. However, mixed results were observed for micronucleus DNA damage assays of commercial formulations (Kier and Kirkland, 2013). Results from DNA damage assays were considered to present solid mechanistic evidence supporting the 2A carcinogenic classification of glyphosate (Guyton et al., 2015).

According to the last review of the glyphosate task force, genotoxic effects may be associated with surfactants present in the formulated products (Greim et al., 2015). Indeed, POEA (a major surfactant used in GlyBH formulations) may for instance be contaminated with 1,4-dioxane, reported at levels up to 300 ppm by the US EPA in 1991 (USDA Forest Service, 2011). 1,4-dioxane caused mammary, liver and nasal cancers in laboratory rodents (Kano et al., 2009). Genotoxic effects of 1,4-dioxane are not clearly established but it acts as a tumor promoter (Stickney et al., 2003); moreover, it had non-linear effects. 1,4-dioxane has never been tested for endocrine effects. The Minnesota Department of Health considered a cancer health risk limit of 1 ppb for 1,4-dioxane (Minnesota Department of Health, 2011). Glyphosate alone also contains carcinogenic contaminants such as N-nitrosoglyphosate (US EPA, 1993; Hebels et al., 2009). Carcinogenicity testing is normally required when the level of nitroso-contaminants exceeds 1 ppm. This is the case in 8% of glyphosate samples, but the US EPA considered that this was not toxicologically significant (US EPA, 1993).

The use of sea urchin embryos, a recognized model for cell cycle studies, allowed the team of Prof. Robert Bellé to identify cell cycle dysfunctions that may be involved in the cancer process. Roundup at 0.8% induced a delay in the first cell cleavage of sea urchin embryos by delaying the activation of CDK1/cyclin B, which controls the cell entry into the mitotic phase (Marc et al., 2002). At a concentration that efficiently impeded the cell cycle, GlyBH inhibited the synthesis of DNA occurring in S phase of the cell cycle (Marc et al., 2004a). These effects were shown to be due to glyphosate acting in synergy with surfactants present in the formulation (Marc et al., 2004b).

The major epidemiological study examining possible associations with pesticide exposures was the Agricultural Health Study, sponsored by the US EPA and the National Health Institute. In this 2005 analysis of 57,311 private and commercial applicators of GlyBH

and among 12 relatively common cancer subtypes, there was a 2.6-fold increased risk of multiple myeloma (95% CI: 0.7–9.4) associated with the use of GlyBH in adjusted analyses (De Roos et al., 2005). However, the statistical relevance of this finding was questioned in a reanalysis funded by Monsanto Europe on the grounds that it is based on a small number of cases (Sorahan, 2015). Since glyphosate may act as a mammary tumor promoter, it is noteworthy that an investigation of breast cancer among farmers' wives found no link with GlyBH use (Engel et al., 2005). Consistent associations were found between non-Hodgkin lymphoma (NHL) and exposure to GlyBH in numerous retrospective population-based case-control studies (Hardell and Eriksson, 1999; McDuffie et al., 2001). A Swedish study (Eriksson et al., 2008) of 910 cases and 1016 controls found a significant excess risk of NHL associated with GlyBH use (OR 2.02; 95% CI: 1.10-3.71). Combining NHL data with those for hairy-cell leukemia (HCL), a rare NHL variant, resulted in a high odds ratio for the risk of GlyBH use (3.04, 95% CI: 1.08–8.52) (Hardell et al., 2002). A similar association between HCL and exposure to GlyBH was reported by Nordstrom (1998) (Nordstrom et al., 1998)(OR 2.9, 95%CI: 1.4-5.9). The most recent systematic review and meta-analysis investigating the association between the incidence of non-Hodgkin lymphoma and occupational exposure to pesticide showed that glyphosate exposure is correlated to B cell lymphoma (OR 2.0, 95% CI: 1.1–3.6) (Schinasi and Leon, 2014). This association with NHL has allowed the classification of glyphosate as a probable human carcinogen by IARC (Guyton et al., 2015).

The results on genotoxicity from epidemiological studies in populations exposed to GlyBH are also controversial, with some claiming positive results (Bolognesi et al., 2009; Paz-y-Miño et al., 2007), but others not (Paz-y-Mino et al., 2011). A recent study revealed DNA damage (increase in micronuclei and nuclear buds) in soybean workers in the State of Rio Grande do Sul (Brazil) (Benedetti et al., 2013). DNA damage (Paz-y-Miño et al., 2007) and chromosomal aberrations (Mañas et al., 2009) were also increased in populations occupationally exposed to GlyBH respectively in Ecuador and Argentina. In the latter, several reports showed increases in incidence of cancers and tumors (Ruderman et al., 2012), especially in the young population (Otano, 2010), but these important findings remain to be properly investigated by epidemiological studies.

Commentary on tumorigenicity and carcinogenicity

Our toxicity study of Roundup chronic effects resulted in a differential mammary tumor incidence between Roundup-treated rats and controls. The design of this study has been widely debated (Séralini et al., 2014b; Séralini et al., 2014c). It was not a carcinogenicity study; in addition, all lethal haemorrhagic tumors were not cancers, and were

not claimed as such in the paper. While the results were considered as not incorrect by a reanalysis of our raw data (Food and Chemical Toxicology, 2014), the tumor findings were considered inconclusive because of the known high incidence of tumors in the Sprague-Dawley rat and the wide normal variability of tumor incidence (Food and Chemical Toxicology, 2014). Indeed, historical data showed that incidences of mammary fibroadenomas among control populations of Sprague-Dawley rats vary from 13 to 62% for mammary fibroadenoma (Giknis, 2004). However, the comparison to historical data enhances control variability and heightens the risk of false negative findings (Cuffe, 2011), because different laboratory rodent diets contain different levels of environmental contaminants that could explain the wide background rate of pathologies in laboratory rodents (Mesnage, Defarge et al., 2015). This is illustrated by the fact that occurrence of some spontaneous neoplasms in historical controls data is not stable over time and is subject to positive or negative time trends (Tennekes et al., 2004).

Evidence of glyphosate effects from epidemiological studies on farmers may be largely biased by the fact that environmental exposure is poorly characterized. In the study of Curwin et al., in 2007, urinary levels of glyphosate were measured among children, mothers, and fathers living in farm and non-farm households. The geometric mean of glyphosate concentration in urine of non-farm and farm children were respectively 2.5 and 1.9 µg/L (Curwin et al., 2007). In general, levels measured in case of occupational monitoring are in the same order compared to environmental monitoring (Niemann et al., 2015). As glyphosate is poorly absorbed by skin or inhalation, glyphosate concentrations reported as occupational exposures may be due to the background of environmental exposures.

In fact, results may largely depend on the test used, the dosage, and the administration route (Heydens et al., 2008). It may also depend on the physico-chemical environment. In the case of the glyphosate metabolite AMPA, Roustan et al. (2014) noted a 20-fold increase in cytogenetic effects after light irradiation of CHO-K1 cells. Cytogenetic effects of glyphosate and AMPA in mixture with atrazine and desethyl-atrazine increased 100-fold after photoactivation (Roustan et al., 2014). Cancer progression may also be promoted at the mitochondrial level following succinate dehydrogenase inhibition that disturbed the epigenetic landscape (Cervera et al., 2009). Interestingly, glyphosate interacts at the succinate binding site of mitochondrial succinate dehydrogenase (Ugarte, 2014).

A definitive answer about GlyBH carcinogenic effects in laboratory animals would come from in vivo carcinogenicity testing at environmentally relevant concentrations. The possibility that glyphosate and Roundup tumorigenic effects may be due to endocrine disrupting effects implies that the 14 carcinogenicity studies performed according OECD guidelines (Greim et al., 2015) could have led to false negative results. Indeed, these studies were performed according to general principles from toxicology, using high doses on adult rodents, but did not incorporate principles from endocrinology. In the latter case, exposure should be started from prenatal life to allow carcinogenic potential to express its effects during the most vulnerable part of development (Soffritti et al., 2008).

6. Reproductive toxicity

Reproductive health is drastically impacted by environmental toxicants (Main et al., 2010; Toppari and Juul, 2010; Toppari et al., 1996). Early puberty, sperm quantity and quality alterations, and congenital malformations are increasingly reported (Benachour et al., 2012). Reprotoxic effects have already been reviewed. Contradictory interpretations have been drawn by authors independent from companies (Belle et al., 2012; Defarge et al., 2012) and by those acknowledging Monsanto for funding (Williams et al., 2012). Few studies have been performed at environmentally relevant levels.

Reprotoxicity has been studied by industry, mostly in multigenerational studies with glyphosate in rats. These were mainly performed according OECD guideline 416. Males of the parental generation were treated during growth, and for at least one complete spermatogenic cycle, and females for at least two complete estrous cycles. The treatment was followed through mating, the resulting pregnancies, and the weaning of F1 offspring. The procedure can be repeated through several generations. Standard observations are for gross signs of toxicities. Additional observations often include the length of the estrous cycle, assays on sperm and other reproductive tissues, and the litter size, viability, and growth of the offspring. Effects on sperm are determined by a number of parameters, e.g., sperm morphology and motility, and histopathology.

Overall, 7 multigenerational studies performed by pesticide companies were considered for glyphosate regulatory assessment. The NOAEL was considered to be approximately 300 mg/kg bw/d for both parental and offspring toxicity (Germany Rapporteur

Member State, 2015). Parental effects in the different studies occurred at a LOAEL of approximately 670 mg/kg bw/d, and toxicity patterns were consistent with symptoms of an acute intoxication (Germany Rapporteur Member State, 2015). These included changes in food and water consumption and lower body weight gain, as well as changes in organ weights. These effects indicate general toxic effects which are probably not due to potential endocrine disrupting properties affecting reproductive performance.

In the peer-reviewed literature, in one study, GlyBH was administered in prepubertal Wistar rats: puberty was delayed and the functions and structure of testes were altered from 5 mg/kg bw (Romano et al., 2010). Some other peer-reviewed studies have exposed rats in utero. In this case, Roundup altered spermatogenesis from 6 mg/kg bw/d and disrupted serum testosterone levels in the adults (Dallegrave et al., 2007). Romano et al. (2012) found that maternal exposure to GlyBH (50 mg/kg bw/d) disturbed the masculinization process and promoted behavioral changes, as well as histological and endocrine problems, with consequences to the reproductive parameters of the progeny.

In human fresh cells or cell lines (table 2), some endocrine-disrupting effects of GlyBH may have been due to inhibition of cytochromes P450. Glyphosate inhibits cytochromes P450 in human cells (Richard et al., 2005), in plants (Lamb et al., 1998), and in rats (Hietanen et al., 1983) at high levels. Glyphosate also disrupts the StAR protein expression in the mouse MA-10 Leydig tumor cell line at 25 ppm after a 2h exposure, which in turn inhibits steroidogenesis (Walsh et al., 2000). Finally, it inhibits testosterone synthesis both in vitro on fresh adult rat isolated testicular cells (Clair et al., 2012) and in vivo in rats (Romano et al., 2010) from 5 mg/kg bw/d, it also disrupted testicular aromatase mRNA levels, among other markers (Cassault-Meyer et al., 2014). In a transcriptomic study in MCF7 mammary human cells, a GlyBH altered estrogen-regulated gene expressions and intracellular responses to hypoxia from 2.3 ppm glyphosate (Hokanson et al., 2007). It also inhibits the transcriptional activities of both androgen and estrogen receptors (Gasnier et al., 2009) from 0.5 mg/L Roundup (containing 0.2 mg/L G). This is however potentially due to a non-specific decrease of global transcription that can be noticed in case of oxidative stress (Berthiaume et al., 2006). Roundup induced oxidative stress and multiple stress-response pathways, leading to Sertoli cell death in prepubertal rat testis (de Liz Oliveira Cavalli et al., 2013). The authors proposed that Roundup provoked a Ca^{2+} overload and a cell signaling misregulation. The cellular stress response and/or the depleted antioxidant defenses could contribute to the Sertoli cell disruption; that could impact spermatogenesis and thus male

fertility (de Liz Oliveira Cavalli et al., 2013). Testicular oxidative stress triggered by glyphosate alone was previously shown in vivo in the same strain of rat (Astiz et al., 2009c).

Interestingly, some studies in non-mammalian species have provided indications of endocrine disrupting effects at more environmentally relevant doses. Changes in ultrastructure and expression of steroidogenic factor-1 were observed in fish ovaries after 15 days exposure to glyphosate at only 65 ppb, a permissible concentration of glyphosate in Brazilian inland waters (Armiliato et al., 2014). Chronic toxicity tests spanning the whole life-cycle on the aquatic invertebrate *Daphnia magna* have shown toxic effects from 50 ppb of glyphosate or Roundup (Cuhra et al., 2013).

A few epidemiological studies have investigated the potential reproductive effects of GlyBH. A reduction of fertility (at least by 20%) was associated with female exposure to GlyBH in the Ontario farm family health study (Curtis et al., 1999). In Columbia, regional differences were noticed in the fertility of populations exposed to GlyBH from aerial spraying for the control of coca plants. However, these effects were not consistent with GlyBH applications (Sanin et al., 2009). Nonetheless, women took longer to conceive in Valle del Cauca (OR = 0.15; 95% CI: 0.12-0.18), a region with historic use of GlyBH. However, as underlined previously, false negative results in these studies may come from the comparison to supposedly non-exposed populations which may have unexpected high levels of glyphosate in their urine.

Commentary on reprotoxicity

The effects of reproductive toxicity are not restricted to a single generation and have thus to be studied across several generations in multigenerational or transgenerational studies. A growing body of evidence indicates that xenobiotics, including pesticides, are able to exert their toxic effects across several generations through epigenetic alterations (Nilsson and Skinner, 2015). It should be underlined that no multigenerational or epigenetic study has been performed with Roundup or its adjuvants, or even with glyphosate at any relevant dose for human/animal exposures. This is an important omission, given that glyphosate is found in the environment at levels within the range of doses known to exercise hormonal activities.

We noticed many conceptual gaps in the current paradigms used to study reprotoxicity. In particular, many authors claim universality for the theory of linear dose-response relationships, the necessity of homogeneity of preclinical toxicological signs in both

sexes in order to take them into account, and the necessity of biochemical disturbances correlated with organ lesions. These concepts, relevant for acute poisoning, are not valid for endocrine disruption (Séralini et al., 2009). Non-linear and sex-specific effects should be considered as potential indications of endocrine disruption rather than as criteria to discriminate false positive results. We also observed that glyphosate reproductive toxicity was not studied with relevant doses (in the range where steroids have an endocrine activity), but at acute toxic doses. Endocrine disruptions may depend on several mechanisms that are not linear to the dose (Vandenberg et al., 2012). Non-monotonic and sex-specific effects have been extensively described for common pollutants acting as endocrine disruptors (Vandenberg et al., 2012). Non-monotonic and sex-specific effects have been reported in many cases with GlyBH, but the regulatory authorities considered these to be false positive outcomes rather than an indication of potential endocrine disrupting effects. False positives may also arise from the use of insensitive models (Myers et al., 2009). For instance, the Charles River Sprague-Dawley CD (CD-SD) rat is widely used, although it is relatively insensitive to exogenous estrogens, including potent estrogenic drugs (vom Saal and Welshons, 2006). Endocrine disruptive effects are thus potentially missed in these studies and they have to be interpreted cautiously. Furthermore, for agents to which all people of all ages may be exposed, exposure should be begun at prenatal life to allow carcinogenic potential to express its effects during the most vulnerable part of the development (Soffritti et al., 2008).

7. Teratogenicity

Embryonic development is one of the most sensitive stages of life for chemical exposure. Evidence of teratogenicity was found in the German authorities' draft assessment report on the industry studies that underlie the authorization of glyphosate in the EU (Antoniou, 2012). The lowest dose of glyphosate alone producing an effect led to the decrease in the mean litter size from 7.7 mg/kg bw/d in a two-generation rat reproductive study (German Federal Agency CPFS, 1998). This was not found in the F2 generation. In a second developmental study, a statistically significantly increased number of fetuses with a dilated heart was found at the lowest dose of 20 mg/kg bw/d, while no fetus was affected in the control group (German Federal Agency CPFS, 1998). After Dr Michael Antoniou's (2012) review (Antoniou, 2012), Monsanto-linked authors answered with a review of seven unpublished rabbit studies in order to determine if glyphosate poses a risk for cardiovascular malformations. (Kimmel et al., 2013). They considered that overall malformation rate or the incidence of cardiovascular malformations was not relevant at dosages below 150 mg/kg bw/d, the point at which severe maternal toxicity was observed, because the effects were not

linearly proportional to dose. Thus, malformations observed from 20 mg/kg bw/d were considered to be a random occurrence.

Among peer-reviewed studies, Dallegrave (2003) found skeletal alterations of Wistar rats treated with a GlyBH at 500 mg/kg bw glyphosate diluted in water. During pregnancy, prenatal exposure to glyphosate disrupted the activity of enzymes related to the energetic metabolism of pregnant rats and their fetuses (Daruich et al., 2001). 1% glyphosate (diluted in water) oral exposure altered liver lipoperoxidation and antioxidant enzymatic systems in 21-day rat fetuses (Beuret et al., 2005). However, the levels of GlyBH or glyphosate used in these experiments are well above human exposure levels.

An increasing number of epidemiological studies have investigated the developmental toxic effects of GlyBH during pregnancy. In fact, increased occurrences of congenital malformations are recorded in South American regions widely dedicated to GM Roundup Ready crop cultivation, where large quantities of GlyBH are sprayed (Benítez-Leite et al., 2009; Campaña et al., 2010; Otano, 2010; Ruderman et al., 2012). Since this topic is of major concern and has arisen relatively recently, surveys are being conducted and have been published initially in governmental and pediatric reports (Vazquez, 2011). Other studies reported increased odds ratios for spontaneous abortions (OR = 1.4; 95% CI: 1.0-2.1) (Arbuckle et al., 2001), miscarriages and preterm deliveries (respectively OR = 1.5; 95% CI: 0.8-2.7 and OR = 2.4; 95% CI: 0.8-7.9 in the crops model) (Savitz et al., 1997), and neural tube defects (OR = 1.5; 95% CI: 1.0-2.4 in a single pesticide model) (Rull et al., 2006).

Commentary on teratogenicity

The teratogenic potential of Roundup became a topic of international debate when Prof. Andrés Carrasco's group published a study of the teratogenic effects of glyphosate in frog and chicken embryos (Paganelli et al., 2010). Embryos appeared to develop cephalic and neural malformations when injected with 8 to 12 µM of glyphosate alone (equivalent to 1.35 – 2.03 mg/L). As with any study showing side-effects of Roundup, this study was debated by the pesticide manufacturers (Saltmiras et al., 2011). The authors replied, but no consensus was obtained (Carrasco, 2011). A link can be made with cytochrome P450 inhibition, a well known endpoint of glyphosate exposure in human cells (Richard et al., 2005), plants (Lamb et al., 1998), and rats (Hietanen et al., 1983) even if the relevance of these mechanisms at environmental levels remains uncertain. A major regulation in the retinoic acid pathways is the degradation of retinoic acid by the CYP26 enzyme. The

inhibition of CYP26 is a coherent explanation of the teratogenic effects of glyphosate alone but remains to be experimentally tested.

Epidemiology findings are not statistically significant or are barely so, and some findings are published in Spanish in non peer-reviewed reports for South American governments. Threats to children's health in Latin America are multifactorial and can be due to indoor and urban air pollution, but also to lead, asbestos, mercury, arsenic, pesticides; hazardous and electronic waste; and even to climate change (Laborde et al., 2015). Nevertheless, taken as a whole, the results are noteworthy. Moreover, similar congenital defects have been reported in piglets contaminated by glyphosate residues due to the consumption of Roundup Ready GMOs (Krüger et al., 2014).

8. General discussion and conclusive remarks

8.1. Adjuvants, contaminants or metabolites also explain the toxicity: differential effects with glyphosate

Differential effects between Roundup and glyphosate (table 1 and 2) are observed in almost all peer-reviewed studies in various mammalian species *in vivo* (Adam et al., 1997; Peixoto, 2005) and in other species (Folmar et al., 1979; Frontera et al., 2011; Moore et al., 2012; Tsui and Chu, 2003). The increased toxicity of the whole formulation in comparison with that of the so-called active principle glyphosate is clearly related to adjuvants. They can be toxic by themselves or facilitate a better uptake of environmental pollutants, and hence increase the body burden of the exposed organism. This is also underlined in reviews sponsored by pesticide manufacturers (Williams et al., 2012). The toxicity of adjuvants appears as a general feature of pesticide toxicology, specifically shown in this major model of the main herbicide worldwide, GlyBH, but also described for other pesticides (Eddleston et al., 2012). Out of 9 major declared active ingredients used in insecticides, fungicides or herbicides, 8 were up to 1000 times less toxic than their formulations on human cells in vitro, and in contrast to the general belief, Roundup was the most toxic of the herbicides and insecticides tested in formulation (Mesnage. et al., 2014). We have summarized the toxic effects of GlyBHs major known adjuvants in table 3.

The composition in adjuvants of different formulations appears to be highly variable (table 3). First, it appears that most of them have been incompletely tested; chronic toxicity

tests as well as information on neurodevelopmental, reproductive and transgenerational effects are generally lacking. In fact, the toxicity of adjuvants or contaminants identified in this investigation is highly variable. Some are relatively safe (sorbic acid, pelargonic acid or glycerine for instance) while others are highly toxic (ethoxylated adjuvants) and even considered to be carcinogenic (for methylparaben, sodium o-phenylphenate, 1,4-dioxane or formaldehyde) and may well be endocrine disruptors by themselves. However, they all have the same toxicological classification when they are included in a pesticide formulation (inert ingredient), but not when they are declared as active ingredients in other products.

We have focused on POEA as a model of GlyBH adjuvant, because it is one of the major surfactants used in GlyBH formulations. It appears that POEA is 10,000 times more toxic than glyphosate in three different human cell lines and is thus a good candidate for secondary side-effects of GlyBH (Mesnage et al., 2013). This finding cannot be attributed only to a phenomenon linked to cell cultures, because POEA also has serious consequences to the health of humans and rats in acute exposures (Adam et al., 1997; Bradberry et al., 2004). POEA toxicity has also been shown in other models, for instance, in vivo in amphibians, crustaceans, fishes, and bacteria (Folmar et al., 1979; Mann and Bidwell, 1999; Mitchell et al., 1987; Tsui and Chu, 2003).

When pesticide residues are found in tap water, food or feed, they arise from the total formulation, and never from the active ingredients only, which are never used alone. High volumes of adjuvants (also called surfactants) are used, and so they (or their breakdown products) can be found in the environment (Berge et al., 2012) and food (Shao et al., 2007; She et al., 2012). Some adjuvants like alcohol ethoxylates can be found in ground water and soil interstitial water collected from farming areas (Krogh et al., 2002) In fact, the half-life of POEA (21-42 days) is even longer than for glyphosate (7-14 days) in aquatic environments (Giesy et al., 2000). Other contaminants like plasticizers can also play the role of adjuvants. This could apply to nonylphenol, a known endocrine disruptor, used as a surfactant in the form of nonylphenol ethoxylates (Vinas and Watson, 2013) and found as a contaminant in the environment (Selvaraj et al., 2014). All the honey, pollen (She et al., 2012) and wax samples monitored in a recent study were contaminated with high levels (up to 10 ppm) of nonylphenol polyethoxylates (Chen and Mullin, 2014).

An exposure to a single formulated pesticide must be considered to be co-exposure to an active principle and the adjuvants. The knowledge of adjuvant toxicity questions the use of glyphosate alone as the only active principle in chronic tests. Regulatory tests should

be also performed with the formulated pesticide to better estimate health risks. We encourage regulators to ask for a complete reassessment of glyphosate formulations rather than glyphosate alone, in particular through a full life-span test on mammals at environmentally relevant doses, with detailed blood and urine analyses, taking into account principles from endocrinology and epigenetics.

8.2. Validity of regulatory assessment

In the US system, the EPA reviews each registered pesticide every 15 years to determine whether it continues to meet Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) standards. Glyphosate's re-registration is expected in 2015. The current approval of the European Union (EU) commission for glyphosate commercialization (2002) is based on a German federal agency review. The next examination of glyphosate was originally scheduled for 2012 but was delayed. Germany (the rapporteur member state) has published its conclusions (German Federal Agency BfR, 2014). Germany has recommended an increase in the ADI from 0.3 to 0.5 mg/kg bw/day, even though more toxicity data have been published since the 2002 approval that cast doubt on the safety of the existing ADI (Fig 2).

Liver and kidneys are clearly affected at doses lower than 350 mg/kg bw/d, which was considered the overall LOAEL in the last draft assessment report. Among studies reviewed, many of them have shown an increased AP activity at various levels. Overall, AP activity generally increases from 10 mg/kg bw/day while statistical significance is reached at different levels. The fact that the raw data is kept confidential prevents any meta-analysis of the chronic toxicity studies results. Among academic studies, biochemical signs of oxidative stress have been detected from 0.09 mg/kg bw/d of glyphosate alone after a subchronic exposure to rats (Larsen et al., 2014; Larsen et al., 2012). After a chronic exposure, a commercial formulation of GlyBH impaired liver and kidneys from 0.1 ppb dissolved in drinking water (Séralini et al., 2014a). Visceral and skeletal malformations arose from 20 mg/kg bw/day in regulatory studies (Antoniou, 2012). All these effects could be explained by the uncoupling effects on oxidative phosphorylation and the inhibition of cytochromes P450 or energy and metabolic enzymes by glyphosate, causing endocrine disruption, oxidative stress and cellular damage, together with possible epigenetic alterations.

In addition, the real and various mixtures of GlyBH to which we are exposed have not been scientifically assessed by regulatory agencies. Adjuvants (such as POEA) amplify the toxicity by increasing glyphosate uptake in cells, or by adding their own toxicity through cell membrane disruption. According to the amplification effects specific to adjuvants and other pollutants in vitro and in vivo, previously described, another safety "mixtures" factor could be applied for GlyBH. The exposure of animals at doses ranging from 1-10 mg/kg bw per day to 5000 or even 10,000 mg/kg bw per day during their whole life is not relevant to conclude on the effects of exposures in the range of 10-100 µg/kg bw per day. Major endpoints of toxicity for both Roundup and glyphosate, such as developmental, reproductive, transgenerational and even chronic effects on adults, still need to be investigated at relevant doses, at which endocrine disrupting effects may arise. The lack of investigation of low dose chronic effects and the neglect of nonmonotonic dose-response relationships make the safety conclusions below 50 mg/kg bw/d of glyphosate questionable. The first and minimal assessment would be to test the chronic toxicity/carcinogenicity of glyphosate at its ADI over the whole life of a mammal, including a prenatal period exposure.

Before awaiting further mandatory and independent chronic assessment of pesticide formulations including Roundup, this large discrepancy should be borne in mind when forming policies for the protection of public health. Overall in the current regulatory assessment, any toxic effect is first suspected to be a false positive, arising by chance, rather than questioning whether no evidence of effect is a false negative result. We encourage regulators to ask for a complete re-evaluation of glyphosate formulations rather than glyphosate alone, taking into account loopholes in the current assessment.

8.3. Toxicity at environmental levels.

Estimations of chronic exposures have been calculated in regulatory assessments by National Estimated Daily Intakes (NEDI) based on the Supervised Trials Median Residue (STMR) of glyphosate, considered to be realistic levels of pesticides found in food. Glyphosate NEDI calculated across various countries was maximal for children in Denmark at 0.0125 mg/kg bw. The German estimation gave a NEDI at 0.0073 mg/kg bw for the general population (14-80 years). Based on limited studies using small cohorts, it is estimated that glyphosate is regularly found in urine at levels corresponding to a dietary daily intake of around 0.1-3.3 µg/kg bw/d (Niemann et al., 2015).

Taken together, studies performed below regulatory limits and relevant for environmental exposures, at best indicate the potential of glyphosate – and more importantly, the commercial formulations containing glyphosate – to cause endocrine related harmful effects at low levels over long periods. At this stage, it is not clear whether this is because of glyphosate, a formulation constituent, or the two together. Drawing any firm conclusion from these studies is not possible at this stage and further work is needed to determine the safety or risk of the herbicide alone or in formulations, especially at levels below the regulatory safe limits and over longer durations. However, glyphosate is never used alone in vivo, and GlyBH formulations have been proven toxic on several cellular and in vivo endpoints below regulatory limits in many studies. This was not the case for glyphosate alone, according to regulatory agencies. With appropriate study design it should be possible to segregate the effects due to glyphosate alone, constituent(s) of the formulation, or the two together. The current evidence presented above raises concerns and indicates the need for further studies. We call for a public, independent, transparent, multidisciplinary assessment of Roundup and other GlyBH.

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10. References

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Figure Legends

Fig 1. Differential effects of Roundup ingredients. Glyphosate, the declared active principle of Roundup, penetrates, and is stabilized because of adjuvants. Glyphosate inhibits aromatic ring synthesis and adjuvants help entry through membranes and to membrane-bound enzymes. This explains its toxic effects in plants through the inhibition of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme (Boocock and Coggins, 1983), but also some endocrine disrupting effects in mammals (Richard et al., 2005). Glyphosate is also a metal chelator and has uncoupling effects on the mitochondrial chain (Olorunsogo et al., 1979). Adjuvants have been found to be more toxic than glyphosate (Mesnage et al., 2013).

Fig 2. GlyBH toxicity is neglected in current regulation. The top line of the figure explains the regulatory tests and assessments driving glyphosate commercial authorizations. Glyphosate has never been tested at the ADI. The middle line represents regulatory limits for glyphosate alone established in the EU. The bottom line represents findings of toxicity below regulatory limits that were dismissed. Neglecting adjuvants has caused regulators to underestimate the toxicity of GlyBH by a factor of 1000 in vitro in an exposure of only 24h (Mesnage et al., 2013).

FIGURE 1

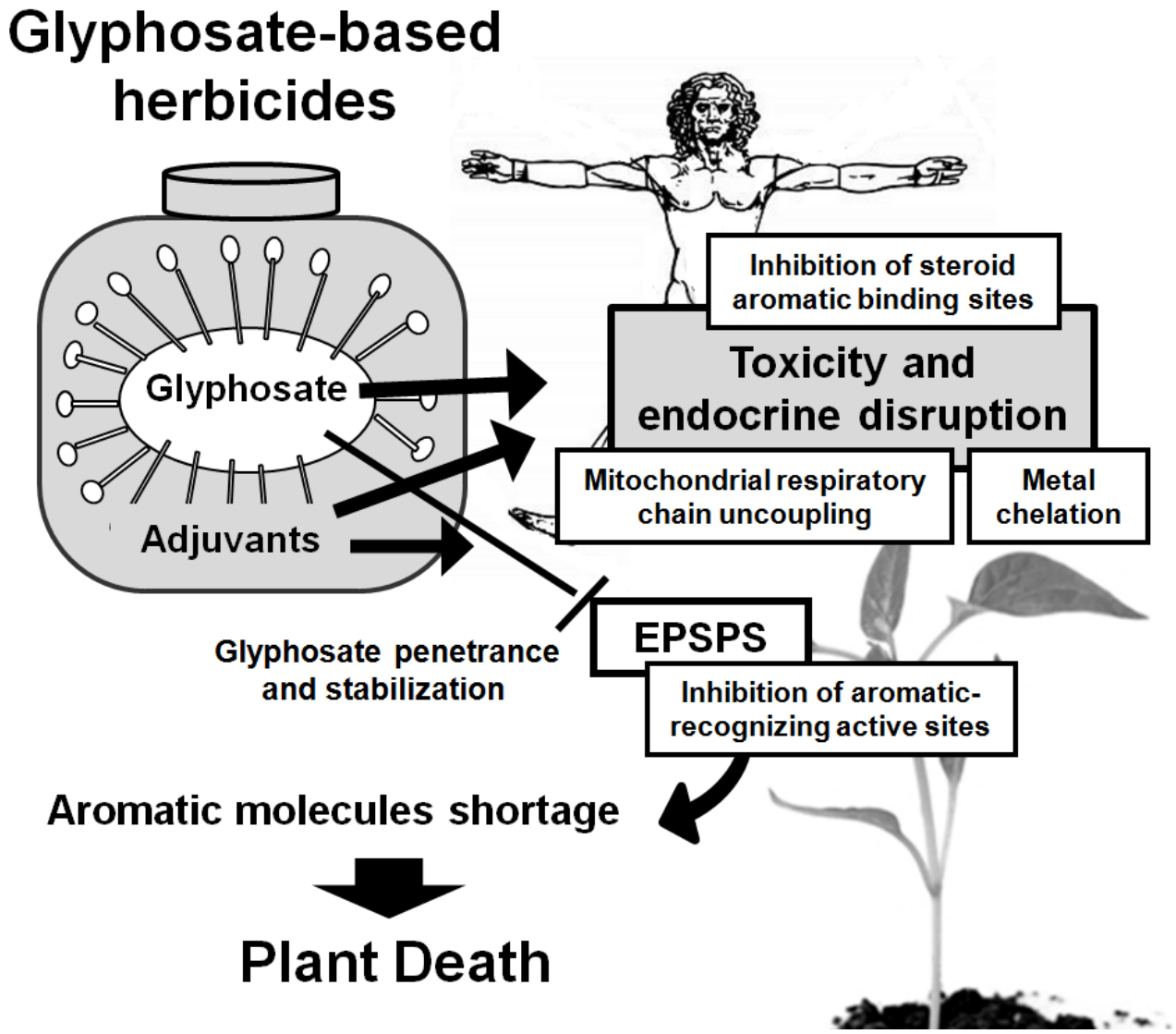
Glyphosate-based herbicides
Glyphosate
Adjuvants
Inhibition of steroid aromatic binding sites
Toxicity and endocrine disruption
Mitochondrial respiratory chain uncoupling
Metal chelation
Glyphosate penetrance and stabilization
EPSPS
Inhibition of aromatic-recognizing active sites
Aromatic molecules shortage
Plant Death

FIGURE 2

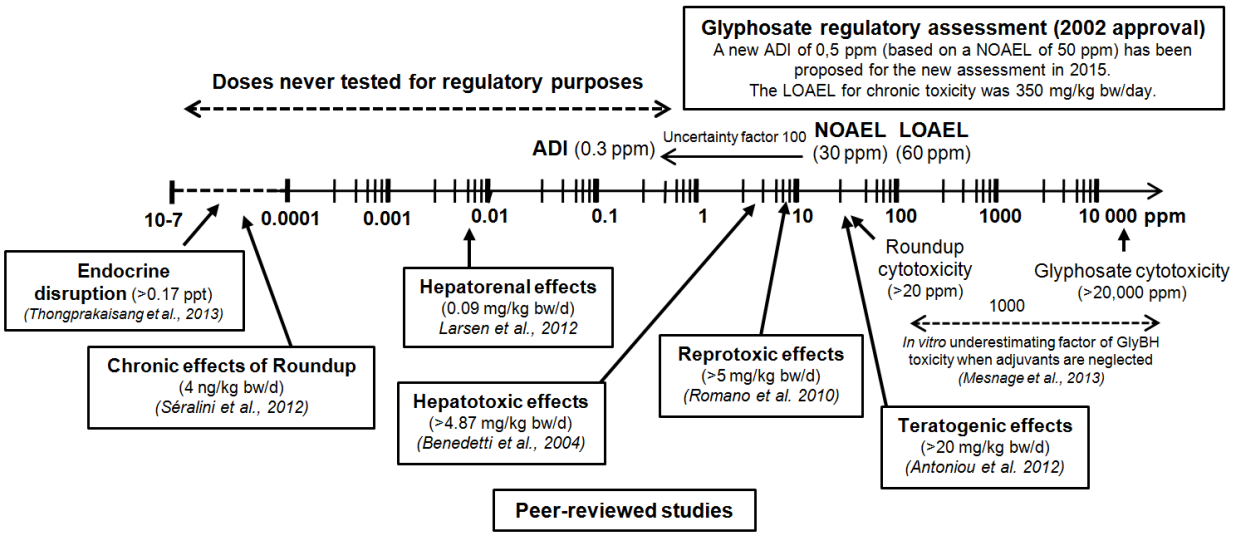
Glyphosate regulatory assessment (2002 approval)
A new ADI of 0,5 ppm (based on a NOAEL of 50 ppm) has been proposed for the new assessment in 2015.
The LOAEL for chronic toxicity was 350 mg/kg bw/day.
Doses never tested for regulatory purposes
ADI (0.3 ppm)
Uncertainty factor 100
NOAEL (30 ppm)
LOAEL (60 ppm)
10-7
0.0001
0.001
0.01
0.1
1
10
100
1000
10 000 ppm
Endocrine disruption (>0.17 ppt)
(Thongprakaisang et al., 2013)
Chronic effects of Roundup
(4 ng/kg bw/d)
(Séralini et al., 2012)
Hepatorenal effects
(0.09 mg/kg bw/d)
Larsen et al., 2012
Hepatotoxic effects
(>4.87 mg/kg bw/d)
(Benedetti et al., 2004)
Reprotoxic effects
(>5 mg/kg bw/d)
(Romano et al. 2010)
Roundup cytotoxicity
(>20 ppm)
Glyphosate cytotoxicity
(>20,000 ppm)
1000
In vitro underestimating factor of GlyBH toxicity when adjuvants are neglected
(Mesnage et al., 2013)
Teratogenic effects
(>20 mg/kg bw/d)
(Antoniou et al. 2012)
Peer-reviewed studies

Table 1. Peer-reviewed published in vivo studies on Roundup and glyphosate toxicology showing toxic effects below regulatory thresholds in mammals. Regulatory limit considered was the LOAEL for chronic effects established at 350 mg/kg bw/day in the last European regulatory assessment. In cases where the mean food intake is lacking, reference conversion factors are applied for mice and rat studies according to EFSA (2012). Most often, peer-reviewed papers only focus on the parameters for which the scientists are specialized and do not study many organs, as is required for regulatory experiments. Doses are expressed in mg/kg bw of glyphosate / day unless otherwise indicated.

Abbreviations: Males, **F**emales; **R**oundup, **G**lyphosate **Species: W**istar rats, **S**prague **D**awley rats; Swiss mice; albino mice; Balb C mice **Time: h**ours**, d**ays**, w**eeks**; Results:** alkaline phosphatase **(AP)**, aspartate transaminase **(ASAT)** and alanine transaminase **(ALAT),** thiobarbituric acid reactive substances **(T-BARS)**, reduced glutathione (**GSH**) and oxidized glutathione (**GSSG**), sum of nitrates and nitrites **[NOx],** Superoxide dismutase activity **(SOD),** Catalase activity **(CAT),** glutathione reductase **(GR),** glutathione peroxidase **(GPx),** Mean Corpuscular Volume **(MCV)**, Mean Corpuscular Hemoglobin **(MCH),** White Blood Cell Count **(WBC),** RED Blood Cell Count **(RBC),** γ-glutamyl transpeptidase (**γ-GT**).

Study	Species (sex)	Product tested	Time	Dose (mg/kg/day)	Results
(Olorunsogo et al., 1979)	**Rat** W (F)	G	5h	15, 30, 60, 120	↗ rate of oxygen consumption by the mitochondria extracted from liver (≥ 60) ↗ of dehydrogenase activities including ATPase activity (≥ 15)
(Benedetti et al., 2004)	W (M)	GlyBH	75d	4.87 – 487 each 2d	*Hepatotoxicity:* ↗ ASAT and ALAT activities (≥ 4.87 mg/kg/2d), ↗ Kupffer cells in hepatic sinusoid and large deposition of reticulin fibers (at 487)
(Dallegrave et al., 2007)	W (F)	R	43d	50, 150, 450	*Reprotoxicity, exposed during pregnancy and lactation* (≥ 50)**:** No maternal toxicity; Adverse reproductive effects on male (↘ sperm number; ↗ abnormal sperm; ↘ serum testosterone; signs of spermatid degeneration and vaginal canal-opening delay; No effects on organ weights
(Astiz et al., 2009b)	W (M)	G alone or combined with zineb and/or dimethoate	5w	10; 3 times a week	*Hepatotoxicity and neurotoxicity:* Liver (L), substantia nigra (SN) and cerebral cortex (CC) - *glyphosate alone:* ↗ m-Calpain activity (SN/CC), ↘ mitochondrial cardiolipin content (SN/CC) - *In combination with other pesticides* (SN/CC/L): inner mitochondrial membrane integrity disrupted, ↗ TBARs, ↘ GSH levels, ↘ mitochondrial cardiolipin content
(Astiz et al., 2009a; Astiz et al., 2009c)	W (M)	G alone or combined with zineb and/or	5w	10; 3 times a week	oxydative stress Liver (L), Brain (B), Kidneys (K), Plasma (P) - *G alone:* ↗ TBARs (all organs); ↗ [NOx] levels (B and P); ↘ total antioxidant ability (P); ↘ Alpha-Tocopherol levels (B and P);↗ glutathione concentration, GSH, GSSG (P); ↘ SOD activity (L and B); ↗ Gamma-glutamyl transpeptidase (P); ↗ CAT (B)

		dimethoate			- *In combination with other pesticides:* ↗ Protein carbonyls (P); ↘ GR (L); ↗ Lactate dehydrogenase (P) *In testis:* ↗ protein carbonyls; No effects on hormonal parameters in plasma or testis
(Astiz et al., 2012; Astiz et al., 2013)	W (M)	G + zineb + dimethoate	5w	G (10) + zineb (15) + dimethoate (15); 3 times a week	*Neurotoxicity:* Lipoic acid prevented oxidative stress and the production of [NOx] caused by pesticides; ↘ glutathione and a-tocopherol levels, ↗ prostaglandins E2 and F2alpha, ↗ calpain activity All restored by lipoic acid. No spontaneous recuperation when the treatment is stopped *Endocrine Disruption*: Parameters restored by lipoic acid: ↗ prostaglandins E2 and F2alpha, ↘ of testosterone production, 3beta- and 17beta-hydroxysteroid dehydrogenases activities, ↗ FSH and LH levels; histological alterations
(Romano et al., 2010)	W (M)	R	30d	5-250	*Endocrine disruption* (after prepubertal exposure): ↗ testis and adrenal weight (250); ↗ Age preputial separation (≥ 50); ↘ seminiferous epithelium height and ↗ luminal diameters (≥ 5) ↘ testosterone (≥ 5) but no effects on estradiol and corticosterone
(Romano et al., 2012)	W (M)	R	GD18-PND5	50	*Endocrine Disruption (gestional maternal exposure):* Changes in sexual partner preference and sexual behavior; ↗ total and daily sperm production; ↗ serum testosterone and estradiol; ↗ LH (pituitary mRNA, protein and serum content); ↗ FSH transcription but no effect on serum FSH or pituitary protein content; ↗ seminiferous epithelium height, ↘ luminal diameter; ↗ Seminal vesicle and epididymis weights; ↘ age and body weight at puberty
(Larsen et al., 2012)	W (F and M)	G	30 or 90d	0.09 or 0.9	*Hepatotoxicity:* No histomorphological changes in liver, kidney and small intestine; ↘ 62 % GST-dependent conjugation (kidney, 90d at 0.9); ↗ hepatic conjugation (+96% at 0.9 and +58% at 0.09); ↗ GPx-dependent reduction (all tissues at 0.9); ↘ TBARS (liver at 0.9 for 90d and in kidney of all rats)
(Caglar and Kolankaya, 2008)	SD (M and F)	R	5 – 13w	56, 560	*Hepatotoxicity:* no differences on the organ weight, food and water consumption; ↗ activity of ALAT, ASAT and on serum lipoprotein (LDL, HDL) and total cholesterol values; No effects on creatinine; large reticulin deposition of hepatocytes and reticular fibril formation.
(Séralini et al., 2014a)	SD (M and F)	R	104 weeks	0.1 ppb, 400 ppm and 0.5% in water	*Chronic toxicity* (≥ 0.1 ppb) *At the end:* ↗ 1.5-2.5 times more mammary tumors leading to death (in females); ↗ 1.4-2.4 times more pituitary dysfunctions (in females); ↗ 3-5.5 times more liver congestions and necrosis (in males); ↗ 1-2 times more kidney severe pathologies (in males) *At the 15th month,* in females: ↗ more kidney dysfunctions (ions leakage observed by biochemical alterations) in females; ↘ Estradiol level; Disruption of testosterone level
(El-Shenawy, 2009)	Albino (M)	R/ G	2w	135 each 2d	*Hepatotoxicity:* ↗ Liver/body weight ratio; For both R and G: ↗ lipid peroxydation, ALAT, ASAT and AP activities, uric acid, urea, nitric oxide, TNF-α, triglyceride and cholesterol; No difference in total protein and albumin; ↘ creatinine and GSH activity.

(Grisolia, 2002)	Swiss (F and M)	R	2d	50, 100, 200	*Genotoxicity (IP):* No effects in the mouse micronucleus test
(Prasad et al., 2009)	Swiss (M)	G	24-72h	25 - 50 mg/kg G	*Genotoxicity (IP)* (≥ 25): ↗ chromosomal aberration; ↘ Mitotic index; ↗ Micronuclei
(George et al., 2010)	Swiss (M)	R	130d	25 mg/kg/day G /2,3d	Tumor promoting potential of G on 7,12-Dimethylbenz(a)anthracene initiated skin carcinogenesis, No initiating potential
(Jasper et al., 2012)	Swiss (F and M)	R	15d	50 or 500	*Hepatotoxicity:* ↗ ALAT, ASAT, and γ-GT levels (both sexes, both doses); ↘ body weight gain (at 50); ↘ body weight (at 500 mg/kg/day); ↘ erythrocytes, hemoglobin concentration, hematocrit (both sexes at 500) ↗ increased MCV characteristic of macrocytic anemia (both sexes at 500)
(Cavusoglu et al., 2011)	Albino (M)	G	3d	50	*Hepatoxicity:* ↗ ASAT, ALAT, blood urea nitrogen, creatinine; ↘ GSH and ↗ malondialdehyde levels (liver and kidney); *Genotoxicity:* ↗ Chromosomal abnormalities, micronuclei and abnormal metaphases. Protective effects of *Ginkgo Biloba*
(Manas et al., 2009a)	Balb-C (F and M)	AMPA	2d	100 -200	*Genotoxicity (IP)* (≥ 100)**:** ↗ Micronuclei. Confirmed by chromosome aberrations in human lymphocytes (1.8mM) and DNA damage in hep-2 cells (≥ 4.5mM)
(Manas et al., 2009b)	Balb-C (F and M)	G	2d	100-400	*Genotoxicity (IP):* ↗ Micronuclei (at 200). ↗ Catalase activity (1h at 400). All was confirmed by DNA damage in hep-g2 cell (≥ 3 mM). No clastogenic effects in peripheral human lymphocyte (0.20-6.00 mM)
(Schiffman et al., 1995)	Gerbil (F)	G	4min	26 – 1690 mg/kg	Alteration of taste response (≥ 100 mg/kg)
(Yousef et al., 1995)	Rabbit (M)	G	6w	38 -380	*Reprotoxicity:* Decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality (≥ 38)
(Cattani et al., 2014)	W (F)	R	GD5-PND15	70	*Neurotoxicity* (immature rat hippocampus): ↘ Na+-dependent glial [3H]-glutamate uptake, glutamine synthetase inhibition, ↗ Ca2+ uptake in hippocampal slices of immature pups
(Larsen et al., 2014)	W (M and F)	R	90d	0.7 ppm in water	*Hepatotoxicity:* ↘ hepatic cytochrome P450 levels and hepatic S-oxidation of methimazole, ↗ carbonyl reduction of menadion, ↗ (F) and ↘ (M) of ethoxycoumarin O-deethylase, ↗ benzyloxyresorufin O-debenzylase and 7-ethoxyresorufin O-deethlyase (F)
(Abarikwu et al., 2015)	W (M)	GlyBH	52d	5 ppm 3 times a week	*Hepatotoxicity:* ↘ GST, ↗ GSH, ↗ lipid peroxidation *Reprotoxicity:* ↘ testosterone level, ↘ sperm count, ↘ sperm motility
(Kumar et al., 2014)	C57BL/6 (F)	G and GlyBH	7d or 3 times a w/3 w	Farm air samples 8.66 ppm	Exposure of air samples collected during GlyBH spray on farms induce IL-33, TSLP and generate IL-13 dependent airway inflammation. G alone failed to produce IL-4.

Table 2. Peer-reviewed published in vitro studies showing differential effects between Roundup or GlyBH and glyphosate. Doses are expressed in mg/L unless otherwise indicated. **Differential effects between GlyBH, Roundup and glyphosate appear in bold characters.**

Abbreviations: Roundup, **G**lyphosate, Catalase activity **(CAT),** thiobarbituric acid reactive substances **(T-BARS)**, reduced glutathione (**GSH**) and oxidized glutathione (**GSSG**), Lethal Dose 50% (**LD50**), inhibitory concentration 50% (**IC50**), γ-glutamyl transpeptidase (**γ-GT**), Superoxide dismutase activity **(SOD),** Catalase activity **(CAT),** glutathione reductase **(GR).**

Author	Species (cells)	Product	Time	Dose (mg/L)	Result
(Walsh et al., 2000)	Mouse (Leydig MA-10)	R/G	2h	10 – 100	*Steroidogenesis disruption* through a post-transcriptional reduction in StAR protein expression ↘ (Bu)2cAMP-stimulated progesterone production (steroidogenesis disruption) **for R not G from 25 mg/L**; ↘ activation of P450scc enzyme activity and 3p-HSD mRNA levels; ↗ StAR transcription and ↘ StAR protein expression distal to PKA activation. Antagonist effects in mixtures
(Axelrad et al., 2003)	Mouse (NB2a neuroblastoma cells)	R / G	24h	See right	*Neurotoxicity:* ↘ of neurite outgrowth **for G at 268 and for R at 1.6 mg/L G + adjuvants**
(Gehin et al., 2005)	Human (HaCaT)	R / G	24-48h	1690 - 4000	*Cytotoxicity:* **IC50 of G and R: 3700 and 3295 mg/L respectively** (24h); Protective effects of vitamin C and E
(Peixoto, 2005)	(Rat) liver mitochondria	R / G	5min	85 – 2535	*Cytotoxicity:* **↘ mitochondrial respiration (≥ 85 mg/L for R, no effects for G)**; ↘ energization and phosphorylation capacities of mitochondria (≥ 169 mg/L for R, not for G); ↘ complex II and III, ATPase, ATPsynthase activities but not complex IV; No effects on mitochondrial swelling; non-specific mitochondrial membrane permeabilization by R
(Potti and Sehgal, 2005)	Human (prostate epithelial cell line PZ-7)	R / G	3d	0.2	*Carcinogenesis:* **R ↗ urokinase plasminogen activator protein expression, not G.** Combined effects with another pesticide
(Richard et al., 2005)	Human (JEG3, placental human) and testicular equine microsomes	R / G	1h	10 - 20000	*Cytotoxicity*: **LC50 of JEG3 (1h) in serum-free medium, for R and G respectively 0.7 and ≥ 2%** *Endocrine disruption* In JEG3: **aromatase activity ↗ in 1h and ↘ in 18h with R (≥ 100 ppm), no effects of G;** ↘ Aromatase mRNA (≥ 200 ppm in 18h); In **microsomes: competitive inhibition (≥ 500 ppm for R and 5000 ppm for G**); Spectral evidence of a direct interaction with the heme of aromatase; Inhibition of reductase (IC_{50} of 5% R)
(Gehin et al., 2006)	Human (HaCaT)	R 3plus / G	24-48h	1690-3380	*Oxidative stress:* **↘ GSH levels (from 1690 mg/L, more with R than G)**; GSSG-Reductase ↗ with 1690 mg/L R; ↗ GSH-Px for high doses; ↘ catalase for 2535 mg/L G

					more than with R; ↗ SOD G at 1690 mg/L more than with R; ↗ TBARs; Protective effect of Vitamin C and E
(Chan et al., 2007)	Rat	GlyBH / G / Adjuvants	20min	169	<u>*Cardiotoxicity*</u>: **Inhibition of the twitch tension (GlyBHand adjuvants) and vasorelaxative effects of rat aorta (GlyBH and G, but more for GlyBH)**
(Martinez et al., 2007)	Human (peripheral blood mononuclear cell)	R / G	24h-96h	0 -2000	<u>*Cytotoxicity*</u>: **IC50 of 56.4 mg/L of G in the form of R and 1,640 mg/L for G (24h).**
(Benachour et al., 2007)	Human (JEG3, HEK293, microsomes)	R Bioforce / G	1-48h	10 - 20000	<u>*Cytotoxicity:*</u> Time dependent ↗ of toxicity (HEK293: LD50 of 0.3% in 1 h to 0.06% in 72h); **Differential toxicity R / G;** Effects delayed by 48h with serum ; <u>*Endocrine disruption*</u>: ↘ Aromatase activity (HEK293, 24h, ≥0.01%) ; No influence of pH but of temperature ; For human placental or equine testicular microsomes, IC50 of 30,000 - 40,000 mg/L of R. Effects from 0.5% (R or G) or human placental microsomes
(Benachour and Séralini, 2009)	Human (JEG3, HEK293, HUVEC)	R / G / AMPA / POEA	6-24h	1 -20 000	<u>*Cytotoxicity:*</u> **Different cytotoxic effect from 20 ppm in 24h, always more than G**; R target the membrane, G mainly cytotoxic by apoptosis at higher levels. AMPA is cytotoxic and the adjuvant is the major toxicant. Combined synergistic effects of the formulations constituents. Fresh HUVEC cells are very sensitive to apoptosis induced by G
(Gasnier et al., 2009)	Human (HepG2, Transfected MDA-MB453-kb2)	R / G	24-48h	0.25 - 20000	<u>*Cytotoxicity:*</u> **Differential cytotoxic effect from 5 ppm in 24h**. ↗ Apoptosis (60ppm). <u>*Endocrine disruption*</u>: ↘ Aromatase activity, ↘ Androgen Receptor mRNA (≥ 0.5 ppm) and Estrogen Receptors mRNA (≥ 2 ppm). <u>*Genotoxicity*</u> ↗ DNA Strand break (≥ 5 ppm).
(Clair et al., 2012)	Rat (Germ cells, Sertoli, their cocultures, and Leydig cells)	R / G	1-48h	0.1 - 10000	<u>*Cytotoxicity:*</u> **Differential cytotoxicity on membrane degradation**; ↗ apoptosis (≥ 100 ppm); Cells still reactive under hCG treatment; No effects on 3beta-HSD Leydig primary cells activity; Inhibition of testosterone synthesis (1 ppm in 24h) ↗ Aromatase mRNA (100 ppm); No effects on estrogen and androgen receptors mRNA
(Koller et al., 2012)	Human (Buccal cell line TR146)	R / G	20 min	10-200	<u>Cytotoxicity:</u> cytotoxicity due to membrane damage and impairment of mitochondrial functions, **LDH release (≥ 10 mg/L for R; ≥ 80 mg/L for G)**; ↗ Apoptosis (≥ 20 mg/L G), <u>*Genotoxicity:*</u> DNA instability and ↗ micronuclei and nuclear buds **(≥ 20 mg/L, more for R than G)**
(Mesnage et al., 2013)	Human (HEK293, JEG3, HepG2)	9 GlyBHs	24h	0.1-10,000	<u>*Cytotoxicity*</u> **:** Different cytotoxic effects from 1 ppm in 24h. **Adjuvants were always more toxic than the formulations, themselves always more than G**.
(Song et al., 2012)	L-929 Fibroblasts, A549 Alveolar cells,	Adjuvants / G	72h	0.066 – 17	<u>*Cytotoxicity :*</u> **Different combined effects with G** TN-20 adjuvant toxicity ↘ in presence of G; LN-10 adjuvant toxicity ↗ in presence of G

	H9C2 heart cells				
(Kim et al., 2013)	Rat (heart H9c2 cells)	G / TN-20 adjuvant	3 – 72h	0.8 – 1.7 G 0.4 – 0.85 TN-20	**Cytotoxicity**: G and TN-20 aggravate mitochondrial damage and induce apoptosis and necrosis. TN-20 seems to disrupt the integrity of the cellular barrier to G uptake, promoting G-mediated toxicity TN-20 but not G ↘ Bcl-2 or Bax expression; Mixed with G at 0.85 mg/L Bcl-2 expression ↘ and Bax expression ↗ as the TN-20 concentration ↗; ↗ Caspases 3/7 expression (0.4 mg/L TN-20 alone or plus 0.8-1.7 mg/L G); Caspases activity decreases at higher TN-20 concentrations because of necrosis; ↘ mitochondrial membrane potential by G when added to TN-20 apoptotic nuclear changes for G alone
(Chaufan et al., 2014)	Human (HepG2)	R / G / AMPA	24-72h		***Differential cytotoxicity (For R and not G)*: ↗ glutathione, ↗ ,superoxide dismutase, ↗ Tyrosine nitration, ↗ caspase 3/7 activity, ↗ nitrosative stress**

Table 3. Overview of the toxicological properties in mammals of known GlyBH adjuvants. Toxicity data are rarely published on these products. Thus the data reported here mainly came from regulatory reports used for the safety assessment of cosmetic products because most pesticide adjuvants are also considered to be generally recognized as safe for uses in cosmetic products. The most widely used surfactants in GlyBH are to date polyoxyethyleneamines (POEA). They are composed of a nitrogen atom bonded to two polyoxyethylene and one long-chain alkylgroup. The latter one derives from tallow, a mixture of animal fats, or from plant fats. Adjuvants have been primarily identified by their declaration on GlyBHs Material Safety Data Sheets (MSDS) extracted from the Monsanto Safety Data Sheets library (http://www.sdslibrary.monsanto.com/Pages/Default.aspx) – the concentration in a specific formulation is given as an example; *, Adjuvants identified by Cox (2004) but not found by our MSDS survey were included; #, GlyBHs contaminants from the manufacturing process.

ADJUVANT / CONTAMINANT	TOXIC EFFECTS
Ethoxylated tallowamine *CAS 61791-26-2*	Toxicity of ethoxylated surfactants mainly due, and proportional, to the ethoxylate chain length (HERA, 2009). Products containing more than 5 and less than 14 ethoxy units appear to be of higher acute oral toxicity.

(~1-18% in different formulations) *(Mesnage et al., 2013a)*	*For the major surfactant used in GlyBHs, ethoxylated tallowamine*: In vitro cytotoxicity from 1-10 mg/L in different human cell types (Mesnage et al., 2013; Szekacs et al., 2014). NOAEL of 36 mg/kg bw/d in a rat suchronic toxicity study based on intestinal irritation at ~100 mg/kg bw/d (Williams et al., 2000). Irritation to the gastrointestinal tract in dogs for CAS 61791-26-2 at 30 mg/kg bw/d, NOAEL was not established (Williams et al., 2000). NOAELs for maternal and developmental toxicity were 15 and 300 mg/kg bw/d, respectively (Williams et al., 2000). No toxicological data were found on chronic toxicity, carcinogenicity, neurotoxicity or endocrine disrupting effects for these specific adjuvants. Extrapolation can be performed based on alcohol ethoxylate studies (HERA, 2009). In a 2-year feeding study with C12-14 alcohol ethoxylate 6.5, the NOAEL was 50 mg/kg bw/d. At the higher dose levels (i.e., 250 and 500 mg/kg bw/d) reduced food consumption and body weight gain was observed. No carcinogenic effect observed with alcohol ethoxylate (HERA, 2009).
Ethoxylated etheralkylamine *(CAS 68478-96-6) (7.5% in Roundup® 450 Turbo)*	
Ethoxylated ether amine *(CAS 71486-88-9) (6% in Amenity Glyphosate 360)*	
1-Propanamine, 3-((C12-C15)alkyloxy) derivs, ethoxylated *(CAS 71486-88-9) (7.5% in Roundup® MAX)*	
Sodium sulphite CAS 7757-83-7 *(<=0.5% in Roundup Max)*	*From (OECD, 2008):* There is no chronic toxicity study available with sodium sulfite. No indication of mutagenic or clastogenic activity up to cytotoxic concentrations. No teratogenic effect in rats, mice and rabbits. No toxicological data were found about carcinogenicity, reproductive toxicity, neurotoxicity or endocrine disrupting effects. Extrapolations based on chronic studies with other sulphites indicates low toxic effects. ADI of 0.7 mg/kg bw (expressed as sulfur dioxide), based on a NOAEL of 72 mg/kg bw/d (expressed as sulfur dioxide) observed in a three generation animal study.
Glycerine CAS 56-81-5 *(<2% in Roundup® Concentrate Weed & Grass Killer Plus FastAct® Select)*	*From (Cosmetic Ingredient Review, 2015; OECD, 2002):* Naturally occurring compound. Repeated oral exposure by gavage does not induce adverse effects other than local irritation of the gastro-intestinal tract. NOEL after a 2-year chronic exposure of rats was 10,000 mg/kg bw (20% in diet). No mutagenic effect (multiple tests) and no carcinogenic effects (at 20% in rat feed for 1 year or up to 10 g/kg for 2 years). Potentiating effect on the carcinogenicity of 4-Nitroquinoline 1-oxide in mice (at 5% oral administration). No effects on fertility and reproductive performance (NOAEL 2000 mg/kg bw) or teratogenic effects (NOEL 1180 mg/kg bw).
Pelargonic acid CAS 112-05-0	*From (Johnson et al., 2011)*: Occurs naturally in many plants. LOAEL of 100 ppm (lower body weight) in short term 28-days toxicity study. LOAEL for teratogenicity greater than 1500 mg/kg bw/d. NOEL for maternal and

(~2% in Roundup® Expres 6H)	developmental toxicity was 1500 mg/kg bw/d. No mutagenic effect. No dermal carcinogenic effects after 50 mg/kg applied twice weekly to interscapular skin on the back of male mice for 80 weeks. No data for oral carcinogenicity. No toxicological data were found about chronic toxicity, neurotoxicity or endocrine disrupting effects.
Polyethylene glycol (5) undecyl ether CAS 34398-01-1 *(~1.5% in Roundup® Expres 6H)*	Reported oral LD50 values in rats: 1710 mg/kg bw (male) and <888 mg/kg bw (female) (NICNAS, 1993). No data found for chronic effects, teratogenic, developmental, neurotoxic or carcinogenic effects.
Bis (2-hydroxyethyl) cocoalkylamine CAS 61791-31-9 *(~4-5% in Roundup® FL 540)*	*EPA Screening level hazard characterization* (EPA, 2010): Reported oral LD50 values in rats ranged from 1200 to >5000 mg/kg bw. Oral dietary studies were performed with rats (two) and dogs (one). In one 90-day rat study, there were gross and microscopic effects in the intestine and lymph nodes at 150 mg/kg bw/d; the NOAEL for systemic toxicity was 50 mg/kg bw /d. In the other 90-day rat study, the NOAEL was 12 mg/kg/d based on decreases in body weight gain and histopathological effects on the intestine and lymph nodes at 400 mg/kg bw /d. The dog 90-day study resulted in a NOAEL of 13 mg/kg bw /d based on clinical signs, decreased body weight and pathological changes in the intestine and lymph nodes at 40 mg/kg bw/d. No data found for chronic effects, teratogenic, developmental, neurotoxic or carcinogenic effects.
Nitroryl, CAS 226563-63-9 *(<3% in Roundup® Flex)*	No toxicological data were found.
Alkylpolyglycoside CAS 68515-73-1 *(<20% in Roundup® Flex)*	LD50 values in rats > 2000 mg/kg bw (Zoller U, 2004). No mutagenic or clastogenic effect. 1000 mg/kg bw/d is considered as the NOAEL for systemic toxicity after suchronic oral exposure. No estrogenic effects detected by the E-screen assay. No teratogenic or developmental effects in rats (100 to 1000 mg/kg bw/d) (Zoller U, 2004). Carcinogenicity studies were not found.
* Methylchloroisothiazolinone CAS 26172-55-4	Contact allergic reaction at permitted concentration in cosmetic (Lundov et al., 2011). Subchronic studies with a commercial methylisothiazolinone/methylchloroisothiazolinone (MIT/CMIT) mixture has evidenced toxic effects on liver and kidneys at 20-30 mg/kg bw/d (Cosmetic Ingredient Review, 1992). Results from genotoxicity studies

	varied between the tests used. Maternally toxic in teratogenicity studies from 1.5 mg/kg bw/day. No treatment-related neoplasms or evidence of systemic toxicity were observed in a carcinogenicity using the MIT/CMIT mixture (Burnett et al., 2010). No studies examining the carcinogenicity of MIT alone were available.
* FD&C Blue No. 1 CAS 3844-45-9	*Data from the SCCNFP assessment (SCCNFP, 2004):* NOAEL for chronic toxicity established at 1072 mg/kg bw/d for males and 631 mg/kg bw/d for females. In a teratogenicity study, an increase in kidney abnormalities (hydronephrosis) in foetuses was seen at 600 mg/kg bw/d. No reproductive toxicity up to 1000 mg/kg bw/day. No gene mutation or clastogenicity in mammalian cells. Carcinogenicity studies showed generally no effects, but the incidence of kidney tumours was increased among male mice that had received a 0.15% dose in one study.
* 3-Iodo-2-propynyl butyl carbamate CAS 55406-53-6	*Data from EPA Reregistration Eligibility Decision (EPA, 1997)*: Acute oral LD50 is 1795 mg/kg and 1056 mg/kg bw in males and females, respectively. Classified as a Group C - possible human carcinogen based increased tumor incidence (mammary fibroadenoma at 20 mg/kg bw/d) in several carcinogenicity studies. Systemic lowest effect level was determined to be 20 mg/kg/day (decreased body weight gain in male rats in a chronic study). In teratogenicity studies, the NOAEL for maternal and developmental toxicity were respectively determined at 20 and 50 mg/kg bw/d. Developmental toxicity was found at 125 mg/kg bw/d (incompletely ossification).
* Light aromatic petroleum distillate CAS 64742-95-6	A one year chronic toxicity study in rats (EPA, 2011) showed signs of liver toxicity such as, increased alanine aminotransferase and total protein in males, increased relative liver [and kidney] weights in females and liver cell hypertrophy in both sexes at the dose of 500 mg/kg bw/d (LOAEL). The NOAEL was 125 mg/kg bw/d. No evidence of genotoxicity (OECD, 2012). In a three-generation reproduction inhalation study in rats, LOAEL on parental generations was 495 ppm (2430 mg/m3) based on reduced body weight (OECD, 2012). The LOAEC for development toxicity was 495 ppm (2430 mg/m3) based on the body weights reductions in the F3 offspring. No data found for carcinogenic effects or endocrine disrupting effects.
* Methylparaben CAS 99-76-3	*Data reviewed by Cosmetic Ingredient Review (CIR) Expert Panel (CIR, 2008):* In chronic toxicity studies, methylparaben induced a reduction of body weight gain when administered at 8% in rat diet during 96 weeks. No toxic effects were detected at 2%. In another study, rats were given subcutaneous injection of 3.5, 2, 1.5, 0.6 mg.kg methylparaben twice weekly during 52 weeks. Mammary fibroadenoma incidence was increased.

	Methylparaben induced the proliferation of mammary MCF-7 cells at a concentration of 5.10^{-5} M. No binding was detected toward estrogen receptors. No teratogenic or male reproductive effects were detected in regulatory studies. In uterotrophic assays, methylparaben produced a significant increase in uterine weight in immature CD1 mice at doses of 103 µmol/kg and above, but not at lower doses of 3.62 and 36.2 µmol/kg. Overall, the NOAEL in uterotrophic assays was 5.5, 5.5, and 16.5 for immature CD1 mice, ovariectomized CD1 mice and immature Wistar rats, respectively.
* Propylene Glycol CAS 57-55-6	Lesions of the lung, heart, liver, spleen, kidney, adrenals and testes in rats chronically administered with 1.23 and 2.45 g/kg bw/d (Fowles et al., 2013). No effects in a comparable study with 0.25, 0.45, 0.95 and 1.9 g/kg bw. No potential for carcinogenicity. NOAEL for developmental toxicity was at least 10.0 mL/kg bw/day. Negligible reproductive or developmental toxicity hazard to human health.
* Sodium benzoate CAS 532-32-1	*Data from OECD SIDS (OECD, 2001):* No adverse effects at < 3145 mg/kg bw (rat 90-day study at 640 to 6290 mg/kg/day). There was increased mortality due to toxic effects in livers and kidneys at 6290 mg/kg bw. Different results were obtained with in vitro genotoxicity assays. Sodium benzoate showed no genotoxicity in vivo. Developmental effects occurred because of maternal toxicity (NOAEL=1400 mg/kg bw/ d, LOAEL = 2800 mg/kg bw/d). No carcinogenic effects were detected in mice (life-long exposure to a 2% solution). No teratogenic effects were detected in 5 studies (doses ranging from 1.75 to 5600 mg/kg bw).
* Sodium o-phenylphenate CAS 132-27-4	Classified as possibly carcinogenic to humans (Group 2B) (IARC, 1999). Tested in mice in one study and in rats in two studies. It induced tumours of the bladder and renal pelvis in male rats in both studies and a marginal increase in the incidence of bladder tumours in female rats in one of the studies. There was no evidence of carcinogenicity in mice. Mixed results were found in assays for genotoxicity in rodents in vivo and in cultured mammalian cells in vitro. A large number of reproduction studies did not show any indication of oestrogen-like or other endocrine effects (Bomhard et al., 2002). No teratogenic effects were observed in rats, mice, and rabbits (Bomhard et al., 2002).
* Sorbic acid CAS 110-44-1	Very low level of mammalian toxicity (Walker, 1990), including acute, short-term and chronic toxicity/carcinogenicity tests, two-generation reproduction and teratogenicity studies. No adverse effects detected

	in chronic studies at up to 10% of the diet.
[#] 1,4 Dioxane *Up to 300 ppm (USDA Forest Service, 2011)*	1,4-dioxane caused mammary, liver and nasal cancers in laboratory rodents (Kano et al., 2009). Genotoxic effects of 1,4-dioxane are not clearly established but it acts as a tumor promoter (Stickney et al., 2003); moreover, it had non-linear effects.
[#] N-nitroso-glyphosate *More than 1% in 8% of samples* (U.S.EPA, 1993).	N-nitroso compounds are genotoxic, carcinogenic to animals, and may play a role in human cancer development (Hebels et al., 2009)
[#] Formaldehyde 2.85% in a filtrate of glyphosate (Smith and Barclay, 1992)	*From EPA Hazard Summary (EPA, 2000):* Acute and chronic inhalation in humans can result in respiratory symptoms and eye, nose, and throat irritation. Association between formaldehyde exposure and lung and nasopharyngeal cancer (human studies). Increased incidence of nasal squamous cell cancer (animal studies). EPA considers formaldehyde a probable human carcinogen (Group B1); IARC consider it carcinogenic to humans (Group 1).