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ECOTOXICOLOGICAL ASSESSMENT OF GLYPHOSATE-BASED HERBICIDES: EFFECTS ON DIFFERENT ORGANISMS

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Abstract: Glyphosate-based herbicides are the most commonly used worldwide because they are effective and relatively nontoxic to nontarget species. Unlimited and uncontrolled use of such pesticides can have serious consequences for human health and ecological balance. The present study evaluated the acute toxicity and genotoxicity of 2 glyphosate-based formulations, Roundup Original (Roundup) and Glyphosate AKB 480 (AKB), on different organisms: cucumber (*Cucumis sativus*), lettuce (*Lactuca sativa*), and tomato (*Lycopersicon esculentum*) seeds, and microcrustacean *Artemia salina* and zebrafish (*Danio rerio*) early life stages. For the germination endpoint, only *L. esculentum* presented significant sensitivity to AKB and *L. sativa* to Roundup, whereas both formulations significantly inhibited the root growth of all species tested. Both AKB and Roundup induced significant toxicity to *A. salina*; both are classified as category 3, which indicates a hazard for the aquatic environment, according to criteria of the Globally Harmonized Classification System. However, Roundup was more toxic than AKB, with 48-h median lethal concentration (LC50) values of 14.19 mg/L and 37.53 mg/L, respectively. For the embryo–larval toxicity test, Roundup proved more toxic than AKB for the mortality endpoint (96-h LC50 values of 10.17 mg/L and 27.13 mg/L, respectively), whereas for the hatching parameter, AKB was more toxic than Roundup. No significant genotoxicity to zebrafish larvae was found. We concluded that AKB and Roundup glyphosate-based formulations are phytotoxic and induce toxic effects in nontarget organisms such as *A. salina* and zebrafish early life stages. *Environ Toxicol Chem* 2016;9999:1–9. © 2016 SETAC

Keywords: Environmental toxicology   Herbicide   Phytotoxicity   Aquatic toxicology   Genotoxicity   Zebrafish   - Roundup

INTRODUCTION

The annual use of pesticides in Brazil has increased alarmingly in recent years [1]. Furthermore, according to the National Health Surveillance Agency (ANVISA), since 2008 Brazil has been the world's largest consumer of pesticides [2–4]. The extensive use of pesticides is justified by improvement in agricultural productivity, which makes the country one of the world's largest food producers. [2]. However, it has been estimated that less than 0.1% of pesticides applied to crops worldwide reach their specific targets, leaving large amounts of toxic residue free to move into different environmental compartments [1,5].

Of the commercial pesticides, glyphosate (N-[phosphono-methyl] glycine)-based herbicide has been the most widely used since the 1970s [6], with an annual application ranging from 0.6 million tons to 1.2 million tons globally [7]. It is used extensively in agricultural and nonagricultural areas to control weeds as a nonselective, broad-spectrum, and postemergent herbicide. Glyphosate is the active ingredient (a.i.) of more than 750 different herbicide formulations. It acts by inhibiting the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme, which is essential for the synthesis of aromatic amino acids in plants, fungi, and some bacteria. Inhibition of EPSPS

causes a shortage of protein and, consequently, plant death. Because the shikimic acid pathway is not found in vertebrates, some scientists and most regulators assumed that glyphosate is safe for mammals, including humans [6,8–11].

Glyphosate is applied in a variety of forms, including isopropylamine salt, ammonium salt, diammonium salt, dimethylammonium salt, and potassium salt. Roundup Original (Roundup), the main commercial glyphosate-based formulation, consists of glyphosate as an isopropylamine salt and a surfactant, polyethylated tallow amine (POEA), which enhances the efficacy of the herbicide [4,8,12]. However, a variety of glyphosate-based formulations, such as Roundup, are registered in more than 100 countries and are available commercially under different brand names [8]. For example, Glyphosate AKB 480 (AKB) is a formulation equivalent to Roundup, composed of 48% w/v of glyphosate as isopropylamine salt, surfactants of unknown composition, and water.

In agricultural fields, glyphosate is sprayed on plant foliage; however, some of the chemical could be deposited directly on the soil surface or carried by the wind to neighboring soils or leach after rainfall to neighboring water bodies, leading to exposure of nontarget terrestrial and aquatic organisms [13]. In Brazil in 2011, approximately 340 million L of glyphosate were sprayed, which caused concern in relation to its possible nontarget effects, especially potential impacts on the health of humans and the ecosystem [14].

Moreover, the development of genetically modified crops has been accompanied by a concomitant increase in the use of glyphosate-based herbicide [13]. The fact that such plants are

tolerant to glyphosate treatment means that repeated applications of these herbicides can be made during cultivation [6]. Currently, genetically modified crops cover 175 million ha in 27 countries worldwide. Of this area, 77% is located in the Americas, with 40% in the United States, 23% in Brazil, and 14% in Argentina [13]. The planting of transgenic soybeans resistant to Roundup has greatly increased the use of this herbicide in Brazilian crops [15].

Peruzzo et al. [16] reported that glyphosate-based herbicide, measured as glyphosate acid equivalent (a.e.), applied to a transgenic soybean crop in Argentina ranged from 0.10 mg a.e./L to 0.70 mg a.e./L in a water sample near cultivated areas. A similar glyphosate level (0.1 mg a.e./L) was detected by Silva et al. [17] in water bodies near areas of intense plantation 30 d and 60 d after the application of glyphosate-based herbicide formulations in southern Brazil. Other studies have reported that the glyphosate concentration in water can reach 1.7 mg/L after direct application of the herbicide [4,18].

Based on official ecotoxicological standard tests, glyphosate can range from being practically nontoxic to being slightly toxic to animals. However, such information cannot be transferred to glyphosate-based herbicides because some of the substances added are occasionally more toxic than the active ingredient itself [13]. According to the Globally Harmonized System of Classification and Labeling of Chemicals, glyphosate (technical grade) is categorized as acute aquatic toxicity 3, hazardous to the aquatic environment, when a fish (96-h median lethal concentration [LC50]), a crustacean (48-h median effective concentration [EC50]), and/ or an algal species (72-h EC50) were used [19]. However, literature data have suggested that the higher toxicity of glyphosate-based herbicides, especially to organisms in the aquatic environment, when compared with glyphosate alone (a.i.) is related to surfactants [13]. The toxicity of Roundup is likely the result of synergistic effects between the glyphosate (a.i.) and the surfactant POEA, which could facilitate glyphosate penetration through plasmatic membranes and consequently potentiate its toxicity action [1,3].

Therefore, the continuous and indiscriminate use of glyphosate-based herbicides and the ability of other formulation components to modify their toxicity have made these products an ecotoxicological concern in the environment. Because glyphosate is tested alone at the regulatory level and because the herbicide formulations differ in terms of amount of glyphosate and inert ingredients, the present study set out to evaluate and compare the acute toxicity and genotoxicity of 2 glyphosate-based herbicides—a reference formulation (Roundup) and AKB—to cucumber (*Cucumis sativus* Linn.), lettuce (*Lactuca sativa* Linn.), and tomato (*Lycopersicon esculentum* Mill.) seeds, and to the microcrustacean *Artemia salina* (Linn.) and zebrafish embryos (*Danio rerio* Hamilton).

MATERIALS AND METHODS

Test substances

Roundup and AKB were purchased from an agricultural supplies retailer. Table 1 shows that both formulations contained isopropylamine salt of glyphosate 48% (w/v) as their active ingredient (480 g a.i./L of formulation) and the equivalent of 36% (w/v) of glyphosate (360 g a.e./L of formulation). The identity of the coformulants declared as inert ingredients is generally maintained as confidential by manufacturers. The inert components added to glyphosate-based herbicides vary between countries and manufacturers [6,20].

Seed germination and root elongation toxicity test

Lettuce (*L. sativa*), cucumber (*C. sativus*), and tomato (*L. esculentum*) seeds, without any chemical additives, were purchased from an agricultural supplies retailer. Selected species are widely cultivated in Brazil and recommended as standard species for ecotoxicological assessment by the US Environmental Protection Agency (USEPA) [21]. Prior to the test, the seeds were sterilized with 10% sodium hypochlorite solution for 5 min and then rinsed several times in distilled water to prevent fungal growth. The seed germination and root elongation test on filter paper was performed according to the USEPA seed germination/root elongation toxicity test [21]. This was carried out separately for each species in Petri dishes. Ten seeds were exposed on filter paper (Whatman filter paper 1) containing 2 mL of the AKB or Roundup formulations at 10 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, 500 mg/L, and 1000 mg/L (equivalent to 3.6 mg/L, 9 mg/L, 18 mg/L, 36 mg/L, 90 mg/L, 180 mg/L, and 360 mg/L of glyphosate, respectively) and controls. Deionized water was used as a negative control and zinc sulfate heptahydrate (at 0.68 mg/L for lettuce, 2.88 mg/L for cucumber, and 4.61 mg/L for tomato) as a positive control. The Petri dishes were closed and incubated in complete darkness in a growth chamber at $25 \pm 1 \, ^{\circ}C$ for 120 h.

After 120 h of exposure, the number of germinated seeds was counted and the length of the root measured. The percentage of relative seed germination was calculated by dividing the number of seeds germinated in the exposed groups by the number of seeds geminated in the negative control. The percentage of relative root elongation was calculated by dividing the mean root length in AKB and Roundup exposures by the mean root length in the negative control.

Table 1. Information regarding glyphosate-based herbicide formulations, Roundup and AKB: Manufacturers, registration number, percentage glyphosate active ingredient, glyphosate acid equivalent, and inert components

Glyphosate-based formulations	Manufacturer (batch no.)	Brazil registration number	IPA salt of glyphosate (% w/v a.i.)	Glyphosate a.e (% w/v a.e.)	Inert components (% w/v)
AKB	Kelldrin Industrial (1156)	325220006[a]	48	36	NI
Roundup	Monsanto of Brazil (BRO011)	0898793[b]	48	36	68.4

[a]National Health Surveillance Agency (ANVISA) [44].
[b]Ministry of Agriculture, Livestock and Supply (MAPA) [45].
AKB = Glyphosate AKB 480; Roundup = Roundup Original formulation; IPA = isopropylamine; a.i. = active ingredient a.e. = acid equivalent; NI = not informed by manufacturer.

The criterion for test validation was that at least 65% of the seeds from the negative control should germinate, and 5 mm of radicular protrusion was regarded as germinated.

Brine shrimp toxicity assay

The brine shrimp bioassay was performed based on the Meyer et al. [22] method and guideline 202 of the Organisation for Economic Co-operation and Development (OECD) [23], with modifications. Brine shrimp (*A. salina*) nauplii were obtained by hatching dehydrated cysts in artificially prepared seawater (3.5% commercial marine salt [Ocean Reef®] in deionized water) at 27 ± 1 °C, under continuous light and aeration for 48 h.

For the test, 20 nauplii, divided into 4 groups of 5 organisms each, were exposed to 2 mL of AKB or Roundup formulations at 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L (equivalent to 1.8 mg/L, 3.6 mg/L, 9 mg/L, 18 mg/L, and 36 mg/L of glyphosate, respectively) and controls. All test solutions were prepared in artificial seawater. Microplates were incubated in the dark in a climatic chamber for 48 h at 27 ± 1 °C. Artificial seawater was used as the negative control and 10 mg/L dodecyl sulfate sodium salt (SDS) as the positive control. After 48 h, the number of dead nauplii (immobility) in the control and exposed groups was counted. The percentage of immobility induced by the formulations was compared with that of the control. In addition, the potential aquatic toxicity classification of AKB and Roundup formulations was determined according to the criteria of the Globally Harmonized Classification System guidance on hazards to the aquatic environment [19].

Fish embryo–larval toxicity test

All organisms used in the fish embryo–larval test were provided by the zebrafish facilities at the Institute of Biology, University of Brasilia, Brazil. The fish were maintained in a ZebTEC (Tecniplast) recirculating system using water obtained by reverse osmosis whereby water passes through several levels of filtration (activated carbon filters and biological filters), is then disinfected by ultraviolet light, and is automatically adjusted for pH and conductivity. The temperature was maintained at 26 ± 1 °C, conductivity at 750 ± 50 µS, pH at 7.5 ± 0.5, and dissolved oxygen equal to or above 95% saturation. Nitrate, nitrite, and ammonia were regularly monitored. This water was used in preparing the test solutions of all assays performed. Adult organisms were fed with commercial dry flake food (TetraMin Tropical Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected approximately 30 min after natural mating, rinsed in water, and examined under a stereomicroscope (Carl Zeiss Stemi 2000-C). Unfertilized or damaged eggs were discarded. The fertilization success was checked, and only batches of eggs with a minimum fertilization rate of 90% were used.

The fish embryo toxicity test was carried out according to OECD guideline 236 [24]. Twenty fertilized eggs per concentration were randomly selected and carefully distributed in a 24-well plate, filled with 2 mL of AKB or Roundup formulations at 5 mg/L, 10 mg/L, 23 mg/L, 50 mg/L, 100 mg/L, 230 mg/L, and 500 mg/L (equivalent to 1.8 mg/L, 3.6 mg/L, 8.3 mg/L, 18 mg/L, 36 mg/L, 83 mg/L, and 180 mg/L of glyphosate, respectively) and controls and negative control (maintenance water). Tests were performed in triplicate in a climate chamber at 27 ± 1 °C and 12 h light under static conditions. Neither food nor aeration was provided during the bioassays.

Embryo development was assessed at 24 h, 48 h, 72 h, and 96 h postfertilization, using a stereomicroscope (Carl Zeiss Stemi 2000-C). In the embryo phase, the following parameters were evaluated: egg coagulation, heart beat presence and tail blood flow, otolith and somite formation, eye and body pigmentation, tail detachment from yolk sac, absorption of the yolk sac, and hatching. Subsequent to hatching, mortality, edema, swim bladder inflation, tail deformities, and undersized embryos were observed and reported.

Alkaline comet assay with zebrafish larvae

The comet assay was performed based on Kosmehl et al. [25] and adapted from Tice et al. [26] in 3 independent series. Surviving zebrafish larvae from the formulation exposure and control groups were pooled and euthanized in ice water. Larvae exposed to sublethal concentrations of AKB at 5 mg/L, 10 mg/L, 23 mg/L, and 50 mg/L (equivalent to 1.8 mg/L, 3.6 mg/L, 8.3 mg/L, and 18 mg/L of glyphosate, respectively) and Roundup at 5 mg/L, 10 mg/L, and 23 mg/L (equivalent to 1.8 mg/L, 3.6 mg/L, and 8.3 mg/L of glyphosate, respectively) were used in the comet analysis. A mechanical cell isolation protocol was used. Twenty pooled larvae were gently disintegrated in 1.5-mL microtubes with a pestle for cell isolation. The cells were resuspended in 1 mL of phosphate-buffered saline (PBS) followed by centrifugation for 10 min at 200 g. The cell suspension was embedded in 100 µL of low-melting agarose 0.7% (w/v) at 37 °C and spread out on slides precoated with 1% (w/v) normal melting agarose. Slides were covered with coverslips and cooled on ice for 10 min. The coverslips were removed, and the slides were incubated in the dark in a lysing solution (100 mM ethylenediamine tetraacetic acid [EDTA], 2.5 M NaCl, 1% [v/v] Triton X-100, and 10% dimethyl sulfoxide [DMSO]; pH 13) at 4 °C for 1.5 h. For DNA unwinding, the slides were transferred to a horizontal electrophoresis tank filled with electrophoresis buffer (12 g/L NaOH and 0.37 g/L EDTA) at 4 °C for 20 min. Electrophoresis was carried out in the same buffer at 4 °C (25 V and 310 mA) for 20 min. The slides were then neutralized with 400 mM Tris-HCl buffer at pH 7.5 for 2 min, and fixed with 100% ethanol for 5 min. They were then stained with ethidium bromide solution (20 µg/mL; Sigma-Aldrich), and examined under a fluorescence microscope (Nikon, model 027012). The DNA lesions were quantified as DNA tail moment using the computer-based image analysis Comet Assay IV on 100 randomly selected cells from duplicate slides.

Statistical analysis

GraphPad Prism® (Ver 5.0, GraphPad Software) was used for the statistical analysis of all assays performed. Comparisons between different experimental exposure groups were performed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Each experimental value was compared with its corresponding control. Statistical significance was accepted at $p < 0.05$.

Toxicity was expressed as effective (EC50) and lethal (LC50) concentrations. GraphPad Prism also calculated EC50 and LC50 values with their 95% confidence limits.

RESULTS

Phytotoxicity

The seed germination and root elongation assay was used to determine the phytotoxicity of the 2 herbicide formulations. The

(A)   **(B)**
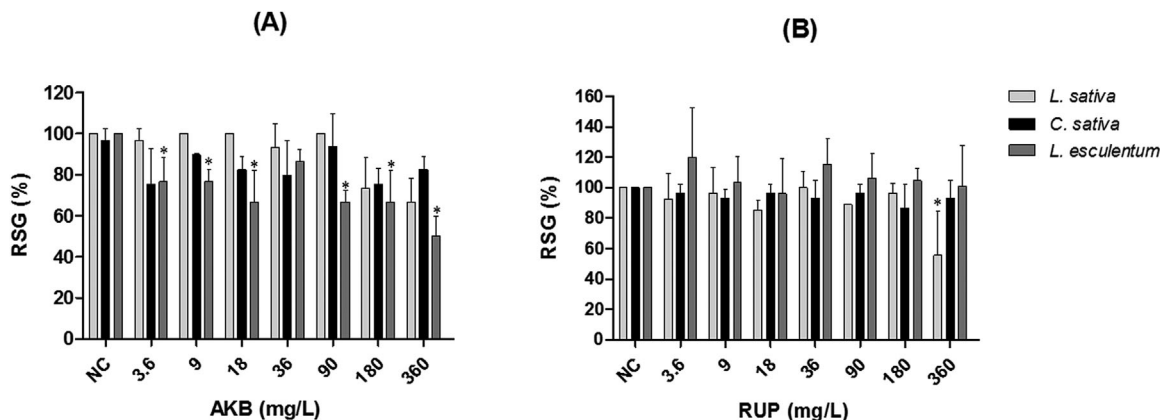

Figure 1. Relative seed germination (RSG) of *Lactuca sativa*, *Cucumis sativus*, and *Lycopersicon esculentum* exposed to different concentrations of Glyphosate AKB 480 (AKB; **A**) and Roundup Original (RUP; **B**) formulations presented as glyphosate acid equivalent. Error bars represent ± standard deviation of 3 replicates. Asterisk (*) represents statistical difference ($p < 0.05$) from the respective negative control (NC).

effects of AKB and Roundup on seed germination of *L. sativa*, *C. sativus*, and *L. esculentum* are presented in Figure 1.

A significant effect ($p < 0.05$) of AKB on seed germination was observed only for *L. esculentum* with an EC50 of 252.80 mg/L of glyphosate acid equivalent (152.30–419.50 mg/L; Figure 1A). In the conditions tested, the lowest-observed-adverse-effect concentration (LOAEC) for this species was 3.6 mg/L of glyphosate (a.e.). The Roundup formulation proved toxic only for *L. sativa*, with an EC50 of 382.70 mg a.e./L (Figure 1B).

Effects of AKB and Roundup formulations on root elongation of *L. sativa*, *C. sativus*, and *L. esculentum* are presented in Figure 2. Both formulations had a significant effect on root elongation of *L. sativa*, *C. sativus*, and *L. esculentum*, and both inhibited the development of all species tested in a dose-dependent manner (Figure 2A and B). The potential toxicity of AKB and Roundup was expressed as the EC50 value (i.e., the concentration that reduces the length of the root; Table 2).

As can be seen in Figure 2, AKB was more toxic to *C. sativus*, with an EC50 of 10.67 mg a.e./L, whereas Roundup was more toxic to *L. esculentum*, with an EC50 of 4.82 mg/L of glyphosate (a.e.). In the conditions tested, the LOAEC for all species exposed to Roundup was 3.6 mg a.e./L (Figure 2B). A similar LOAEC value was observed for *C. sativus* and

L. esculentum seeds exposed to AKB, whereas the value was 18 mg a.e./L for *L. sativa* (Figure 2A).

Brine shrimp acute toxicity

Figure 3 shows the percentage mortality of *A. salina* nauplii after 48 h of exposure to different concentrations of AKB and Roundup formulations. Control groups had 100% viable organisms (mobility).

Both AKB and Roundup induced significant toxicity to *A. salina* nauplii in dose- and time-dependent ways (Figure 3). The mortality ratio was expressed using the corresponding LC50 values to determine the toxicity of the formulations for this marine microcrustacean (Table 3). According to the Globally Harmonized System of Classification and Labeling of Chemicals, both herbicides are classified as category 3 for acute aquatic toxicity; however, Roundup was found to be more toxic to *A. salina* nauplii (48-h LC50 of 14.19 mg a.e./L) than AKB, which had a 48-h LC50 of 37.53 mg/L of glyphosate acid equivalent (Table 3).

Zebrafish early life stages acute toxicity

Mortality of zebrafish embryos and larvae was recorded at 24 h, 48 h, 72 h, and 96 h. Both herbicides induced a concentration- and time-dependent increase in mortality. In Figure 4, the dose–response curves clearly show that Roundup

(A)   **(B)**
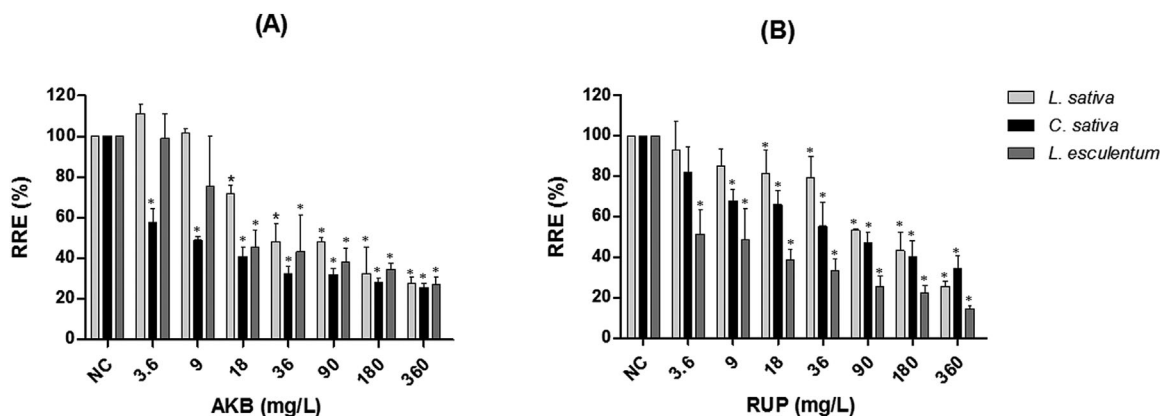

Figure 2. Relative root elongation (RRE) of *Lactuca sativa*, *Cucumis sativus*, and *Lycopersicon esculentum* exposed to different concentrations of Glyphosate AKB 480 (AKB; **A**) and Roundup Original (RUP; **B**) formulations presented as glyphosate acid equivalent. Error bars represent ± standard deviation of 3 replicates. Asterisk (*) represents statistical difference ($p < 0.05$) from the respective negative control (NC).

Table 2. Median effective concentration (EC50) on root elongation of *Lactuca sativa*, *Cucumis sativus*, and *Lycopersicon esculentum* after 120 h exposure to AKB and Roundup formulations

Glyphosate-based formulations	EC50 (mg/L)[a]		
	L. sativa	*C. sativus*	*L. esculentum*
AKB	72.43 (53.34–98.34)	10.67 (7.71–14.78)	40.25 (24.91–65.04)
Roundup	60.80 (45.79–80.74)	71.12 (46.82–108.00)	4.82 (2.97–7.82)

[a]Confidence intervals shown in parentheses.
AKB = Glyphosate AKB 480; Roundup = Roundup Original formulation.

is more toxic than AKB for *D. rerio* in all exposure periods. This fact is confirmed with LC50 values for AKB and Roundup at 24 h, 48 h, 72 h, and 96 h (Table 4). Zebrafish death occurred at concentrations of AKB that were approximately 1.5- to 2.7-fold higher than the LC50 of Roundup.

The hatching rate (percentage of hatch) determined by counting the zebrafish larvae outside the eggshell is presented in Table 5. Both formulations induced premature hatching of zebrafish eggs compared with negative control at 48 h, with EC50 values of 6.23 mg/L (3.16–12.28 mg/L) for AKB and 8.29 mg/L (3.05–22.53 mg/L) for Roundup.

Zebrafish larvae genotoxicity

Figure 5 shows the degree of DNA strand breakage (measured by DNA tail moment) in single cells derived from zebrafish embryos after exposure to the 2 herbicides and controls for 96 h. There was no significant genotoxicity to zebrafish larvae exposed to sublethal concentrations of AKB and Roundup compared with the negative control.

DISCUSSION

Environmental risk assessment is based on knowledge of the toxic effects of contaminants [27]. Considering that herbicides can be deposited on the soil surface during agricultural practices, it is important to elucidate their effects on the physiological processes related to plant growth to understand the mechanisms at work and their possible effects on nontarget plants [15].

In the present study, we evaluated the phytotoxicity of the AKB and Roundup glyphosate-based formulations to 3 seed species (*L. sativa*, *C. sativus*, and *L. esculentum*) in a worst-case exposure scenario (above environmentally relevant concentrations). We observed that both formulations caused a weak

alteration in the germination rate of *L. esculentum* and *L. sativa*, with EC50 values (252.80 mg a.e./L for AKB and 382.70 mg a.e./L for Roundup) much higher than the maximum concentration of glyphosate found in the environment (1.7 mg/L). Thus, this parameter might not be a sensitive endpoint for the toxicity assessment of glyphosate-based formulations.

In contrast, root elongation seems be a sensitive endpoint for toxicity assessment of AKB and Roundup because both formulations inhibited the development of all species tested with EC50 values measured as glyphosate acid equivalent of less than 100 mg/L. Both Roundup and AKB inhibited the growth of *L. esculentum* and *C. sativus* roots, with EC50 values of 4.82 mg a.e./L and EC50 of 10.67 mg a.e./L (approximately a 3-fold to 6-fold higher environmentally relevant concentration), respectively.

Although glyphosate is not recommended for direct application to soil, it can reach the ecosystem via foliar wash-off and undirected spray drift contamination and by exudation from roots or death and decomposition of treated plant residues [28,29]. Once in the soil, glyphosate may be adsorbed onto soil particles, degraded by microbes, or transferred to deeper soil horizons, migrating via soil pores or root canals [15].

Recent studies have suggested a risk of glyphosate toxicity to nontarget plants as a result of the rhizosphere transfer of glyphosate. Glyphosate has been shown to affect plant physiological mechanisms such as photosynthesis, C metabolism, mineral nutrition, and oxidative events, and to disturb plant–microorganism interactions [15].

The soil half-life of glyphosate varies from a few days to 2 mo or 3 mo, but there are some reports of soil persistence for 100 d and 1000 d, and persistence of the phytotoxic activity for more than 19 wk after application [28]. Microbial degradation is considered the most significant transformation process for determining the persistence of herbicides in the soil [9]. This process is carried out in both aerobic and anaerobic conditions by the microflora found in the soil. The primary metabolites are glyoxylate and aminomethylphosphonic acid, which eventually degrade to water, carbon dioxide, ammonia, and phosphate [9,15].
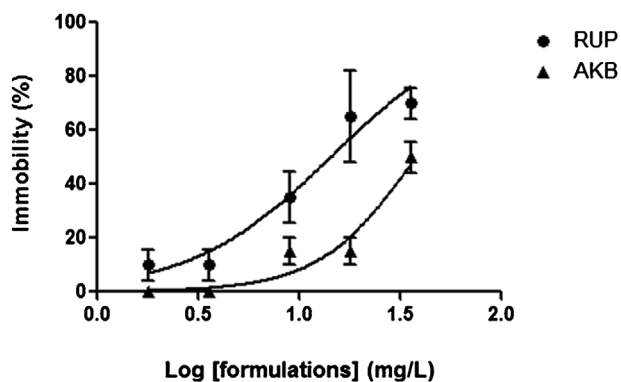

Figure 3. Sigmoid dose–response curve showing the effects of the Glyphosate AKB 480 (AKB) and Roundup Original (RUP) formulations on the mortality of *Artemia salina*.

Table 3. Median lethal concentrations (LC50) causing 50% mortality of *Artemia salina* after 48 h exposure to the AKB and Roundup formulations, and toxicity classification according to Globally Harmonized System of Classification criteria[19]

Glyphosate-based formulations	LC50 (mg/L)[a]	Toxicity classification
AKB	37.53 (30.40–46.33)	Category 3
Roundup	14.19 (9.64–20.88)	Category 3

[a]Confidence intervals shown in parentheses.
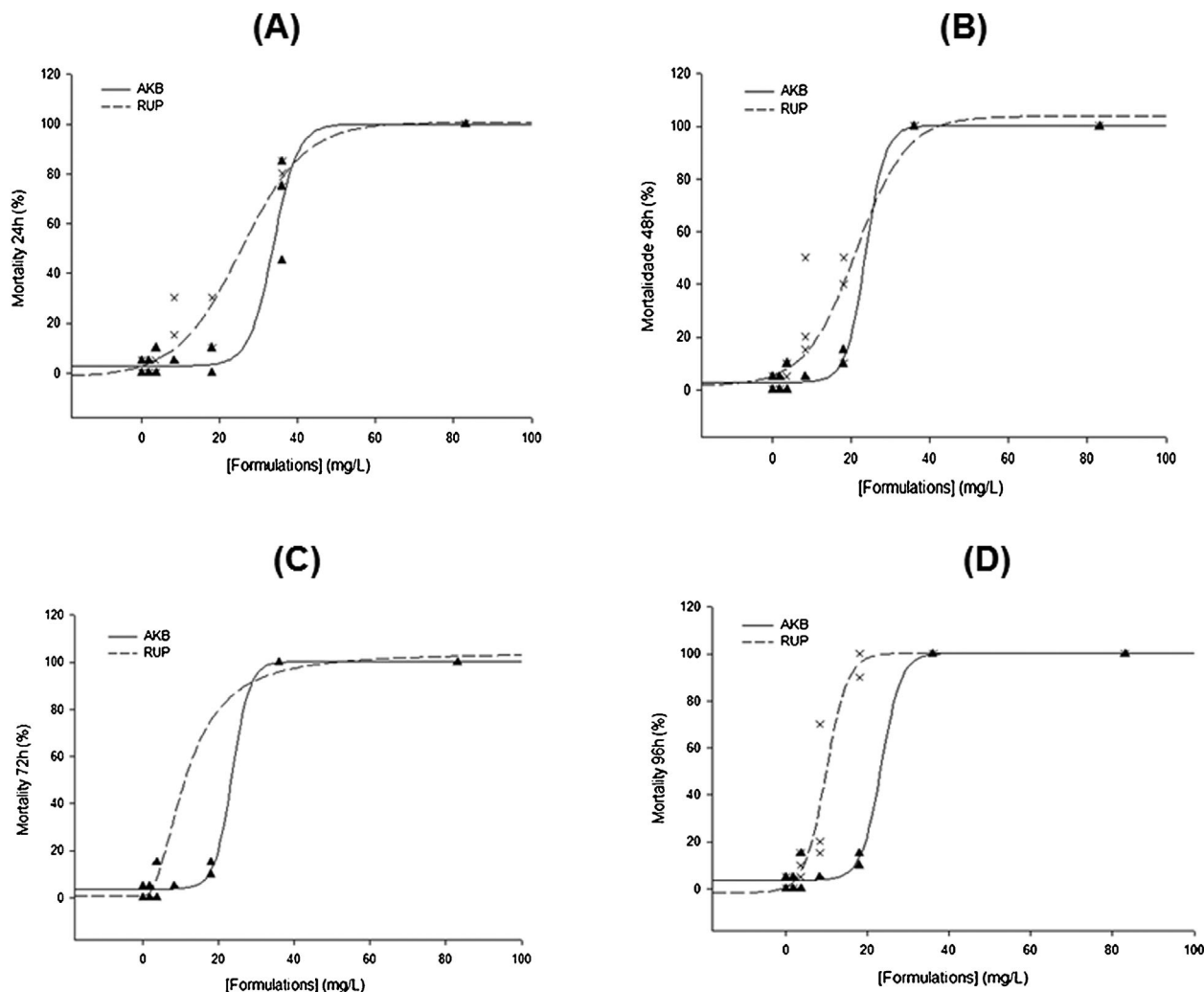AKB = Glyphosate AKB 480; Roundup = Roundup Original formulation.

Figure 4. Dose–response curves showing the effects on mortality of zebrafish embryos and larvae after 24 h (**A**), 48 h (**B**), 72 h (**C**), and 96 h (**D**) of exposure to Glyphosate AKB 480 (AKB) and Roundup Original (RUP) formulations. The triangle and × symbols represent the dispersion of the data obtained during exposure to AKB and RUP, respectively, in 3 independent replicates.

Aquatic contamination by herbicides can occur as a result of direct spraying, or during heavy rainfall or leaching of agricultural fields [30,31]. Glyphosate has been regularly detected in a diversity of water bodies [32], and its presence in surface waters has been found 60 d after the formulation was applied, which indicates that this compound can persist in the environment [17,30]. Moreover, up to approximately 0.04 mg a.e./L has been reported to occur in rivers near urban runoff and wastewater treatment effluent. Therefore, exposure to nontarget organisms is inevitable and is related to glyphosate's high water solubility [30].

Our results showed that AKB and Roundup induced significant toxicity to *A. salina* nauplii; and although they presented toxicity classifications similar to those of the Globally Harmonized System of Classification and Labeling of

Table 4. Median lethal concentrations (LC50) causing 50% mortality of zebrafish embryos/larvae at different exposure times to the AKB and Roundup formulations

Exposure time	AKB LC50 (mg/L)[a]	Roundup LC50 (mg/L)[a]
24 h	48.13 (24.48–94.62)	27.39 (17.17–43.69)
48 h	27.98 (12.65–61.91)	17.50 (9.54–32.12)
72 h	27.64 (12.53–60.97)	10.80 (5.73–20.35)
96 h	27.13 (12.48–58.99)	10.17 (5.16–20.03)

[a]Confidence intervals shown in parentheses.
AKB = Glyphosate AKB 480; Roundup = Roundup Original formulation.

Table 5. Effects of AKB and Roundup formulations on hatching success of zebrafish embryos[a]

Formulation	48 h (mean ± SE)	72 h (mean ± SE)	96 h (mean ± SE)
AKB (mg/L)			
NC	27.1 ± 7.2	100 ± 0	100 ± 0
1.8	67.8 ± 1.4*	100 ± 0	100 ± 0
3.6	62.4 ± 6.5*	100 ± 0	100 ± 0
8.3	66.6 ± 1.7*	98.2 ± 1.7	98.2 ± 1.7
18	70.0 ± 10.1*	96.3 ± 3.7	98.1 ± 1.8
Roundup (mg/L)			
NC	26.7 ± 7.2	100.0 ± 0	100.0 ± 0
1.8	73.3 ± 6.0*	100.0 ± 0	100.0 ± 0
3.6	53.3 ± 16.4	100.0 ± 0	100.0 ± 0
8.3	48.3 ± 16.8	100.0 ± 0	100.0 ± 0
18	60.0 ± 8.8	100.0 ± 0	100.0 ± 0

[a]Values represent the hatching rate (% hatch) mean ± standard error (SE).
*Statistically different ($p < 0.05$) from the respective negative control (NC).
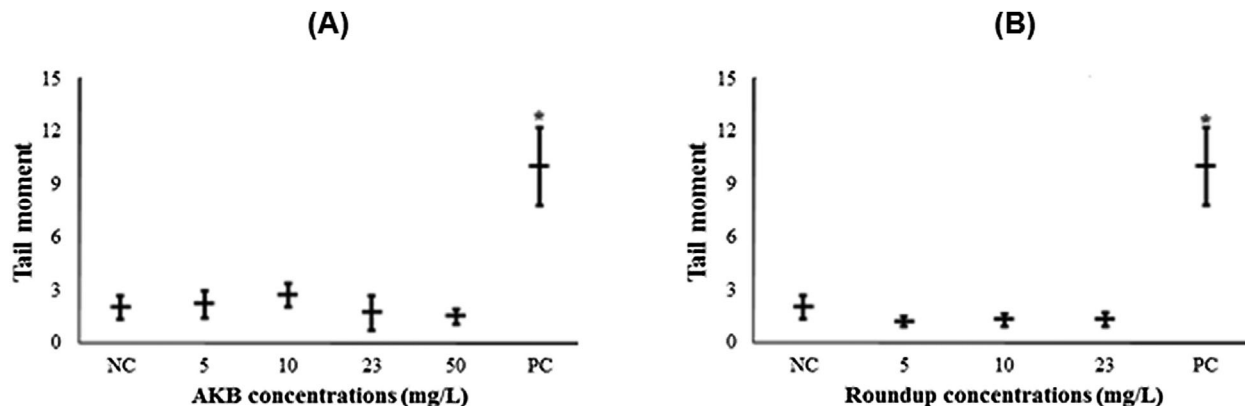AKB = Glyphosate AKB 480; Roundup = Roundup Original formulation.

(A)

(B)

Figure 5. Genotoxicity following exposure of whole embryo to Glyphosate AKB 480 (AKB; **A**) and Roundup Original (RUP; **B**) in the comet assay with zebrafish cells. Data are given as tail moment values. For each concentration tested, 100 cells in 3 independent assays were investigated. Asterisk (*) represents statistical difference ($p < 0.05$) from the respective negative control (NC). PC = positive control (ultraviolet light for 5 min).

Chemicals (category 3), the marine microcrustacean was more sensitive to Roundup than to AKB. According to the literature, Roundup shows low toxicity to nontarget marine organisms, with LC50 values ranging from 10 mg/L to 100 mg/L of glyphosate active substance [32,33]. A similar value was observed in the present study; AKB was toxic for *A. salina*, with a 48-h LC50 of 37.53 mg a.e./L, whereas Roundup had a 48-h LC50 of 14.19 mg/L for this marine microcrustacean. In contrast, Tsui and Chu [12] showed toxicity for *Acartia tonsa* and *Ceriodaphnia dubia,* marine and freshwater invertebrates, with LC50 values of 1.77 mg/L and 5.39 mg/L, respectively, after 48 h of exposure to Roundup formulation (commercial grade with 41% a.i.).

An analysis of contamination levels and identification of the compartments where this herbicide accumulates can help to guide ecotoxicological studies [9]. However, there is little information available comparing the effects of commercial glyphosate-based formulations on different organism levels. Most of these studies compare the toxicity of technical-grade glyphosate with its commercial products and show that the formulation has a higher toxicity than the technical-grade glyphosate. Commercial glyphosate formulations vary in composition with country and purpose, and the properties of these formulations, including their toxicity, can be compared by using the concentration of glyphosate present, expressed as glyphosate acid equivalent [20].

Glyphosate-based herbicides are generally consist of approximately 36% to 48% glyphosate, water, salts, and adjuvants such as POEA. Glyphosate is never used without its adjuvants, which enable and enhance its herbicidal activity by promoting its toxicity. Adjuvants are considered and declared inert diluents because they are not held to be directly responsible for the pesticide activity and are classified as confidential for regulatory purposes. However, the fact that an ingredient of a mixture is active in plants does not mean a priori that this ingredient is the most toxic of the mixture, either for humans or for other levels of biodiversity [6].

Despite its widespread use, neither glyphosate nor its various formulations are routinely monitored in surface waters, and nontarget organisms could experience direct toxic effects from such formulations or be indirectly affected by changes to ecosystems or food resources. Our results showed that both AKB and Roundup are toxic to the embryonic stages of zebrafish; however, AKB is less toxic than Roundup, the most

widely used glyphosate-based formulation in the world. Several authors have suggested that the toxicity of this formulation may be derived from synergistic effects between glyphosate and other formulation products, such as a surfactant that enhances the penetration of glyphosate through the plant cuticle (e.g., POEA) [7]. Folmar et al. [34] compared the acute toxicity of technical-grade glyphosate acid, Roundup, isopropylamine salt of glyphosate, and POEA to several freshwater invertebrates and fishes. The authors observed that acute toxicity of the surfactant and Roundup formulation were similar (96-h LC50 ranging from 2.3 mg/L to 43 mg/L), whereas technical glyphosate (96-h LC50 of 140 mg/L) was considerably less toxic than Roundup or surfactant.

Both AKB and Roundup affected the chorion of embryos and induced considerable premature hatching in zebrafish embryos, at 6.23 mg/L and 8.29 mg/L, respectively. Various chemical and other environmental stressors, such as temperature, are known to affect developmental rate and, subsequently, hatching time. In a study by Webster et al. [35], zebrafish embryos presenting results similar to 10 mg/L Roundup had also hatched prematurely.

There are no data in the literature on the genotoxicity of glyphosate-based formulation for embryos and fish larvae. The comet assay has been described as a sensitive and valuable tool for detecting DNA damage in adult fish [36–39]. Glyphosate-based formulations have been associated with inducing oxidative stress in several adult fish during biotransformation [36–40] and as the inhibiting antioxidant enzyme. As the antioxidant defense is incapable of neutralizing oxidative stress, DNA damage could be induced, as observed in several studies [36–39].

Although glyphosate was classified as noncarcinogenic in humans (group E) in 1991 by the USEPA, the International Agency for Cancer Research recently considered the significant findings from the USEPA report and several more recent positive results and concluded that glyphosate should now be classified as a carcinogenic substance in group 2A (probably carcinogenic to humans), based on limited evidence in human experiments and sufficient evidence in animal experiments [41].

The results of the present study showed that sublethal concentrations of Roundup and AKB were not able to promote genotoxic effects in zebrafish larvae. Although there is a strong correlation of zebrafish embryo to fish acute toxicity, the low sensitivity of embryos to genotoxicity induced by glyphosate

contained in the formulations could be associated with a lack of metabolic capability at this life stage, as suggested by Knobel et al. [42] during an investigation of ethanol genotoxicity, which requires metabolic activation.

Monitoring programs should be implemented, as evaluation of glyphosate has not often been included in regular monitoring programs because of their costly methods, which results in a long-term deficiency in global datasets [43].

CONCLUSIONS

Although the target mechanism of action of glyphosate and glyphosate-based formulations is specific to plants, we concluded that AKB and Roundup formulations are phytotoxic and induce toxic effects in nontarget organisms such as *A. salina* and zebrafish early life stages. It is important, therefore, to assess not only the ecotoxicity of the active ingredient but also of the formulations to protect the environment and prevent damage to human life.

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