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Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms

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ABSTRACT

Glyphosate (GLY) is the active ingredient of several herbicide formulations widely used to control weeds in agricultural and non-agricultural areas. Due to the intensive use of GLY-based herbicides and their direct application on soils, some of their components, including the active ingredient, may reach the aquatic environment through direct run-off and leaching. The present study assessed the acute toxicity and genotoxicity of the GLY-based formulation Atanor 48 (ATN) and its major constituents GLY, surfactant polyethoxylated tallow amine (POEA), as well as the main metabolite of GLY aminomethylphosphonic acid (AMPA) on non-target aquatic organisms. The toxic effects of these chemicals were evaluated in the fish embryo acute toxicity test with zebrafish (*Danio rerio*), while genotoxic effects were investigated in the comet assays with cells from zebrafish larvae and rainbow trout gonad-2 (RTG-2). GLY and AMPA caused no acute toxic effect, while ATN and POEA induced significant lethal effects in zebrafish (LC_{50}-96 h 76.50 mg/L and 5.49 mg/L, respectively). All compounds were genotoxic in comet experiments with zebrafish larvae (LOEC 1.7 mg/L for GLY, ATN, AMPA and 0.4 mg/L for POEA). Unlike *in vivo*, only POEA induced DNA damage in RTG-2 cells (LOEC 1.6 mg/L), suggesting that it is a direct acting genotoxic agent. In summary, these data indicate that the lethal effects on zebrafish early-life stages can be ranked in the following order from most to least toxic: surfactant POEA > formulation ATN > active ingredient GLY ≈ metabolite AMPA. Genotoxic effects were observed in both RTG-2 cells (only POEA) and zebrafish (all test compounds) with the lowest tested concentrations. Therefore, it is important to evaluate different toxicological endpoints as well as use different non-target organisms to predict the hazards of GLY-based formulations and their components and breakdown product to aquatic biota.

1. Introduction

Since 2008, Brazil occupies the first position in the world ranking of countries that consumes pesticides [1,2]. These compounds have significant economic, environmental and public health impacts [3]. The widespread use of pesticides in Brazil is related to the high agricultural productivity, making this country one of the world's largest food producers [1]. In addition, Brazil is the world's third largest agricultural exporter, behind the United States of America and European Union [4]. The high agricultural productivity of Brazil supported by pesticide use has been associated with a variety of human health problems and environmental impacts [5].

Glyphosate [N-(phosphonomethyl) glycine; GLY] is currently among the most widely used herbicides worldwide [2,5,6]. GLY is the active ingredient of several commercial formulations used to control weeds in agricultural and non-agricultural areas (*e.g.*, domestic use) [7–9]. The broad-spectrum herbicidal activity of GLY in combination with the use of genetically modified crops has exponentially enhanced the use of GLY-based formulations [6,10–13]. In agricultural fields, GLY is sprayed on plant foliage, but it can reach water resources, mainly

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through soil run-off or leaching processes, leading to exposure of non-target aquatic organisms [14–18]. In water bodies, GLY-based herbicide measured as GLY acid equivalent has been detected from 0.01 to 0.7 mg/L, reaching the worst-case for surface waters of 1.7 mg/L [4,15–17].

GLY has no or low toxicity to non-target organisms, because the shikimic acid pathway is not verified in animals [6,19–21]. However, GLY-based formulations have been associated with toxic effects in animals, which have been attributed to the presence of surfactants such as polyethoxylated tallow amine (POEA) [5,6,13,16,22–24]. GLY is never used alone in agriculture because may not penetrate or concentrate enough in plants to exert herbicidal activity. It has been suggested the addition of POEA could facilitate the penetration of GLY through animal cell membranes, as it does for plant cell [5,13]. Consequently, there is evidence that the bioconcentration factor of GLY is increased with the presence of POEA on aquatic environments [25,26].

In addition, GLY has also been detected together with its major breakdown product aminomethylphosphonic acid (AMPA) in plants, soil, and water [13,27,28]. According to Food and Agriculture Organization (FAO), AMPA is of potential toxicological concern, mainly due to its accumulation in the food chain [28].

Faced with the above, this study was realized to assess the acute toxicity and genotoxicity of the GLY-based formulation ATN and its major constituents, namely active ingredient GLY, surfactant POEA, as well as the main metabolite of GLY AMPA on zebrafish (*Danio rerio*) early life-stage and rainbow trout gonad-2 cell fish line (RTG-2). Fish are also currently used in the assessment of chemical toxicity in aquatic environments, since they are the most diverse group of vertebrates found in this ecosystem [30]. Zebrafish early life-stage test has been recommended as an alternative for acute toxicity tests with juvenile/adult fish. This model identifies a series of morphological and behavioral endpoints which are used to predict the mode-of-action of chemicals [31–33]. Similarly, fish cell line RTG-2 derived from gonads of the rainbow trout (*Oncorhynchus mykiss*) has been successfully introduced for detection of genotoxic effects as an alternative to *in vivo* fish bioassays [33–36].

2. Material and methods

2.1. Test chemicals

Technical-grade glyphosate (GLY; Glyphosate PESTANAL®; purity 99%, CAS No. 1071-83-6) and aminomethylphosphonic acid (AMPA, purity 99%, CAS No. 106651-9) were purchased from Sigma-Aldrich. GLY-based formulation Atanor 48 (ATN), composed of isopropylamine salt of glyphosate at 48% (w/v) as active ingredient (or 480 g a.i./L of formulation) and 36% (w/v) of glyphosate equivalent (or 360 g a.e./L of formulation), was obtained on agricultural supplies retailer. In this study, ATN exposure is expressed as glyphosate acid equivalents (a.e.). The identity of the co-formulants declared as inert ingredients is, in general, maintained as confidential by manufacturers. Polyethoxylated tallow amine (POEA; CAS No. 61791-26-2; purity 64%) was purchased from Crescent Chemical Company.

2.2. Zebrafish maintenance and egg production

Adult male and female zebrafish (*D. rerio*) were provided by the zebrafish facility (ZebTec Tecniplast) at the Institute of Biology, University of Brasília and kept in separate tanks (ethical approval UFG Nº 102/2014). Fish were maintained in a Rack Hydrus (Alesco) recirculating system using water filtered by reverse osmosis, where water passes through several levels of filtration (activated carbon filters and biological filters), is then disinfected by ultraviolet (UV) light and 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 of 8 ppm. 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 (TetraColor Flakes®) and live brine shrimp. On the day of the test, zebrafish eggs were collected about 30 min after natural mating, rinsed in water and examined under a stereomicroscope (Bel Photonics STM PRO). 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.

2.3. Fish embryo acute toxicity (FET) test

The zebrafish embryo-larval toxicity test was carried out according to OECD Test Guideline 236 [29]. Twenty fertilized eggs per concentration were randomly selected and carefully distributed in a 24-well plate, filled with 2 mL of GLY, ATN, AMPA at 1.7, 5, 10, 23, 50 and 100 mg/L and, POEA at 0.4, 0.8, 1.6, 4, 8 and 16 mg/L and controls (negative control – NC: maintenance water and positive control – PC: 3,4-dichloroaniline at 4.5 mg/L). Tests were performed in triplicates (three independent experiments) in a climate chamber at 26 ± 1 °C and 12 h light under static conditions. Neither food nor aeration was provided during the bioassays. Embryo development was assessed at 24, 48, 72 and 96 h post-fertilization (hpf), using a stereomicroscope (Bel Photonics STM PRO) with 3x magnification. The distinction between the normal and abnormal development of embryos was established according to the zebrafish development descriptions reported by Kimmel et al. [37]. Lethal (egg coagulation, no somite formation, non-detachment of the tail from yolk sac and no heart beating) and sublethal (effects on the eye and body pigmentation, absorption of the yolk sac, hatching rate, swimming bladder inflation, otolith, presence of edemas and blood accumulation, tail deformities) parameters were observed and reported.

2.4. Alkaline Comet assay with zebrafish larvae

The Comet assay was performed based on Kosmehl et al. [30]. and adapted from Tice et al. [38]. Surviving zebrafish larvae (10–20 larvae per concentration) from the GLY, POEA, ATN and AMPA exposure and control groups (NC: maintenance water and PC: UV light for 10 min) were pooled and euthanized in ice water. The larvae exposure to UV light was conducted at 320 nm for 10 min in maintenance water [33]. Their nuclei were isolated using trypsin/EDTA at 0.25/0.03% (v/v) for 10 min at 37 °C. Then a trypsin neutralizer was added and centrifuged at 212 g for 10 min. The cells were resuspended in phosphate-buffered saline (PBS, pH 7.4) and filtered using a cell separator (100 μm). The cell suspension was embedded in 80 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 lysing solution (100 mM EDTA, 2.5 mM NaCl, 1% sodium dodecyl sulfate, 1% Triton X-100, and 10% dimethyl sulfoxide: 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 (0.8 V/cm 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 (DMI 4000 B, Leica Microsystems, Bannockbum, USA). Determination of damage was carried out by visual scoring and classification into five categories based on tail intensity and length, from 0 (no tail) to 4 (almost all DNA in tail) [39–41]. The total score expressed as genetic damage index (DI) was calculated by the equation: DI = [(nº nucleoid class 0) x 0] + [nº nucleoid class 1) x 1] + [(nº nucleoid class 2) x 2] + [(nº nucleoid class 3) x 3] + [(nº nucleoid class 4) x 4], on 100 randomly selected cells from duplicate slides [42]. The experiments were

performed in triplicate and the length of DNA migration measured in 100 randomly selected nucleoids (50 per slide).

2.5. In vitro alkaline Comet assay with the rainbow trout gonad-2 cell line (RTG-2)

RTG-2 cells (5×10^4 cells/well, 24-well plate) [European Collection of Authenticated Cell Cultures (ECACC) 90102529], cultured at 22 °C L-15 (Leibovitz-15) medium with 10% fetal bovine serum, 1% L-glutamine and 1% antibiotic (Penicillin-Streptomycin) (all from Gibco®), were exposure to GLY, ATN and AMPA (10, 23, 50 mg/L), and POEA (1.6, 4, 8 mg/L) for 3 h. Untreated cells (*i.e.*, cells cultured at L-15 medium) was used as NC, while cells treated with 0.5 mM methyl methanesulfonate (MMS, CAS No. 66-27-3, Sigma-Aldrich) was used as PC. After 3 h of exposure, the RTG-2 cells which presented cell viability ≥ 80% (Trypan Blue Dye Exclusion Test) were harvested and processed for the alkaline Comet assay (pH > 13) as described by Felzenszwalb et al. [33] and Oliveira et al. [36] DNA lesions were quantified as tail intensities using a computer-based image analyzer (Metafer CometScan v.2.8.0®, Metasystems, Germany) on 100 randomly selected nucleoids per treatment. The *in vitro* Comet data represent three independent experiments (*i.e.*, three biological replications) with single well per treatment.

2.6. Statistical analysis

The FET and Comet data were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Each experimental value was compared to its corresponding negative control and the statistical difference was considered significant when $p < 0.05$. With respect to the FET, the toxicity was expressed as the lethal concentration (LC_{50}), which was calculated using GraphPad Prism software (version 5.0, GraphPad Software, San Diego, CA, USA) with 95% confidence interval.

3. Results

3.1. Acute effects for zebrafish early-life stages

The present study investigated the effects of the GLY-based formulation ATN, active ingredient GLY and its metabolite AMPA, as well as of the surfactant POEA, on the zebrafish embryonic development (survival and malformations) at 24, 48, 72 and 96 h of exposure.

According to Fig. 1, no significant mortality was observed in zebrafish early-life stage after exposure to different concentrations (1.7–100 mg/L) of GLY (Fig. 1A) and AMPA (Fig. 1D), which presented survival rate ≥ 90% in all exposure periods. In contrast, POEA (Fig. 1B) and ATN (Fig. 1C) induced significant lethal effects to zebrafish with LC_{50}-96 h of 5.49 mg/L and 76.50 mg/L, respectively. POEA was the most toxic compound, exhibiting concentration- and time- dependent toxicity. In addition, all embryos exposed to 16 mg/L of POEA were coagulated in the first 24 h of exposure ($p < 0.001$). ATN was more toxic than active ingredient GLY, inducing significant mortality at the highest tested concentration (100 mg/L; $p < 0.001$) from 48 h of exposure.

In relation to sublethal effects, Fig. 2 shows that GLY (E–H), POEA (I–L) and ATN (M–P) induced some morphological abnormalities, including: pericardial and yolk sac edemas, spinal curvature, head and tail deformities in different exposure times; however, these malformations were not statistically significant when compared to their respective negative control.

3.2. Genotoxicity test

The DNA damages were estimated using two fish test systems – zebrafish early-life stages and RTG-2 cell line (gonadal cell line) – in order to verify potential species- or organ-specific responses.

Fig. 3 shows the levels of DNA strand breaks measured by DNA damage index (DI) in single cells derived from zebrafish larvae after 96 h of exposure to GLY, POEA, ATN, AMPA and controls. Our findings demonstrated that all tested compounds induced genotoxic effects from the lowest tested concentrations with LOEC of 1.7 mg/L for GLY, ATN, AMPA and 0.4 mg/L for POEA. GLY (Fig. 3A) induced the highest levels of DNA damage ($p < 0.0001$) followed by POEA (Fig. 3B; $p < 0.001$), ATN (Fig. 3 C; $p < 0.001$) and AMPA (Fig. 3D; $p < 0.05$).

Unlike *in vivo* assay with cells from zebrafish larvae, wherein the results pointed out genotoxicity for all tested compounds, Fig. 4 shows that only POEA induced DNA damage in RTG-2 cells in a concentration-dependent manner (inverse effect; $p < 0.05$) (Fig. 4D) with LOEC of 1.6 mg/L, suggesting that it is a direct acting genotoxic agent.

4. Discussion

As previously mentioned, the direct application of GLY-based formulations on the soil can result in aquatic contamination by their constituents (active ingredient GLY and surfactant POEA) and GLY metabolite AMPA through direct run-off, mainly during heavy rains, and leaching [14,24,43].

GLY is usually detected in the aquatic environment with its major metabolite AMPA [13,27,28]. There is a lack of knowledge for concentration levels of AMPA in waters bodies, as well as for the toxic potential of this compound to aquatic biota [27,28]. However, it is known that AMPA has higher persistence and mobility in soils compared to GLY [6,40], and thus its ecological risk is of note [28].

Our current results showed that GLY and AMPA did not induce acute toxicity in zebrafish early-life stage with LC_{50}-96 h > 100 mg/L. Similar effect was observed by Fiorino et al. [44]. in assessing the acute effects of GLY (0.005; 0.05; 5; 10 and 50 mg/L) on early-life stages of zebrafish and common carp (*Cyprinus carpio*) for 120 h. The authors demonstrated that all tested concentrations, except the highest concentration (50 mg/L), induced cumulative mortality ≤ 10% after 96 h of exposure. GLY at 50 mg/L caused the highest cumulative mortality, reaching 17.5% after 120 h of exposure [43] while in our study, there were no significant differences between this group (GLY at 50 mg/L) and control with 1.7% of larvae mortality after 96 of exposure. It is worth noting that according to OECD 236 [29], the survival of embryos in the NC must be ≥ 90% (validation criterion of the test), and therefore mortality ≤ 10% in the experimental groups is acceptable. In addition, several studies suggest that the levels of GLY residues in water (ranging from 0.01 mg a.e./L to 1.7 mg a.e./L) [15–17] are not capable of causing toxicity in aquatic species, but the toxicity can be highly species dependent [6]. Fiorino et al. [44] demonstrated that GLY from 5 mg/L in 96 h induced significant cumulative mortality in common carp (*C. carpio*). Therefore, there still is a need in improving the knowledge about the impacts of GLY on aquatic environment [28].

The adoption of genetically modified crops increased substantially the use of GLY-based formulation due to the acquired resistance that requires several re-applications of this herbicide [10,45]. There is a discrepancy when the toxicity of the active ingredient GLY is compared with the toxicity of the GLY-based commercial formulations [6]. GLY-based formulation components are found in water because the active ingredient GLY is never used without its adjuvants, which allow and enhance its herbicidal activity, facilitating penetration of the active principle GLY into the plant cuticle [20,46]. Although there is a variety of adjuvants, the most used surfactant in GLY-based herbicide formulations is the polyethoxylated tallow amine (POEA) [6]. It has been found in superficial and ground water collected from agricultural areas and its half-life (21–42 days) in aquatic environment is even longer than GLY (7–14 days) [20].

POEA toxicity has traditionally been assessed by comparing with active ingredient GLY or by evaluating POEA at nominal concentrations due to difficulties in determining the environmentally relevant

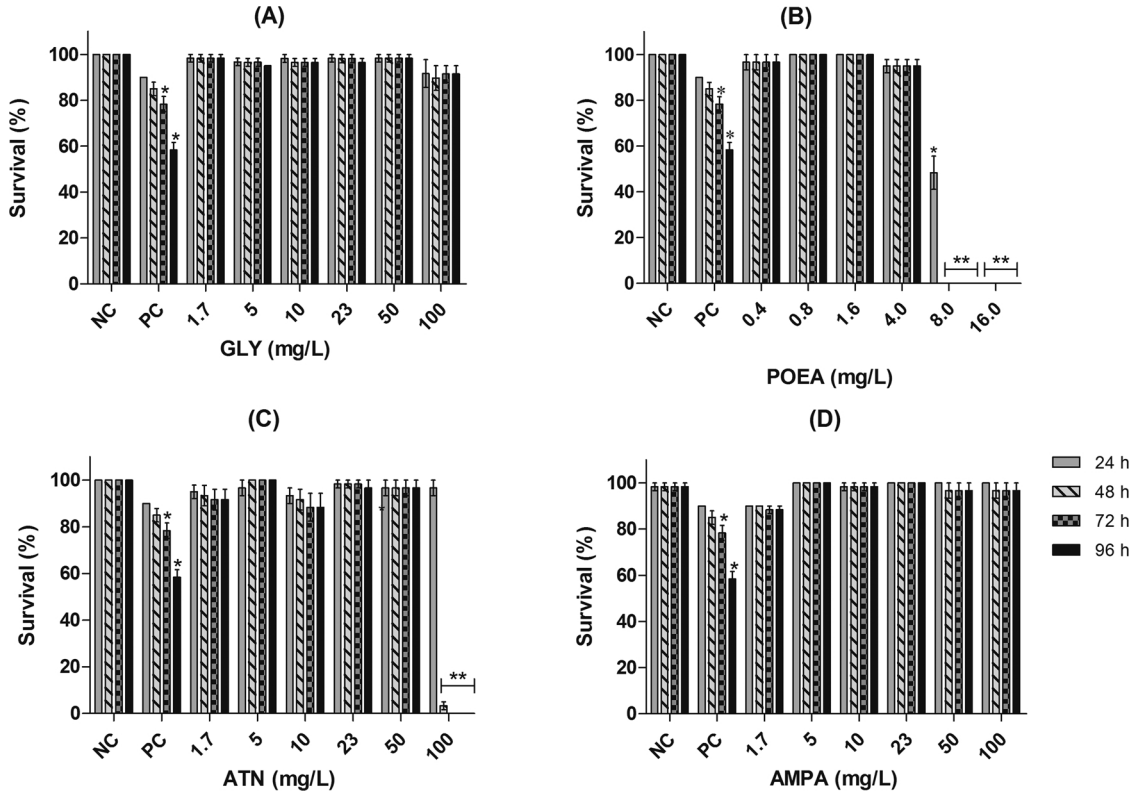

Fig. 1. Survival rate of zebrafish at different developmental stages exposed to GLY (A), POEA (B), ATN (C) and AMPA (D) for 24, 48, 72 and 96 h. Twenty fertilized eggs per experimental group were evaluated. Bars represent the mean ± standard error of the mean of three independent experiments. *$p < 0.05$ and **$p < 0.0001$ statistically different from the respective negative control (NC) based on one-way ANOVA and Dunnett's post hoc test. PC = positive control (3,4-dichloroaniline at 4.5 mg/L after 24, 48, 72 and 96 h of exposure).

concentrations of this surfactant [25]. Perkins et al. [47] evaluated the effects of the GLY-based formulations Rodeo® (GLY active ingredient formulated without a surfactant) and Roundup® (a reference formulation with GLY formulated with a surfactant) on the embryonic development of *Xenopus laevis* using Frog Embryo Teratogenesis Assay-Xenopus (FETAX). They observed that Rodeo® was the least toxic, with a LC_{50} of 5407 mg a.e./L, while Roundup® showed LC_{50} of 9.4 mg a.e./L. The authors also revealed that POEA, surfactant of the Roundup®, showed LC_{50} of 2.7 mg/L. Several authors have suggested that the toxicity of commercial formulations may be derived from synergistic effects between GLY and other formulation products, such as the surfactant POEA [48].

In our study, we compared the potential toxicity of ATN (GLY-based formulation equivalent to Roundup), POEA and GLY alone on the embryonic development of zebrafish. Some of our results are in agreement with Perkins et al. [47], showing that POEA is the most toxic constituent of GLY-based formulation (LC_{50}-96 h of 5.49 mg/L) followed by the ATN formulation (LC_{50}-96 h of 76.50 mg/L), GLY alone and its metabolite AMPA (LC_{50}-96 h > 100 mg/L), for which the acute toxicity was not observed. Other data from our group showed that both Roundup® and GLY AKB 480 (formulation equivalent to Roundup®) were toxic to the embryonic stages of zebrafish; however, AKB (LC_{50}-96 h of 27.13 mg/L) and ATN (investigated in this study) were less toxic than Roundup® (LC_{50}-96 h of 10.17 mg/L) [16].

Concerning the alterations in zebrafish morphology, we observed that the embryos and larvae exposed to GLY (from 10 mg/L to 100 mg/L), POEA (from 0.4 mg/L to 8 mg/L) and ATN (50 mg/L and 100 mg/L) caused some malformations or delayed development which were not relevant (no significant statistical differences; $p > 0.05$).

In this work, we also evaluated the potential of GLY, POEA, ATN and AMPA in inducing primary DNA lesions on fish, using comet assay with zebrafish larvae and RTG-2 cells. All tested compounds were genotoxic for zebrafish larvae with GLY inducing the highest level of DNA damage from the environmentally relevant concentration (1.7 mg/L; $p < 0.05$) [15–17]. It is also important to note that the DNA damage induced by POEA occurred at concentrations lower than those of the other tested compounds. Studies have pointed out that POEA is more toxic than the active ingredient GLY and the formulated products [49].

Similar to our results, Navarro et al. [49] demonstrated that POEA at 0.15, 0.75 and 1.5 mg/L induced DNA damage in blood cells of *Prochilodus lineatus* with scores of DNA damage significantly higher ($p < 0.001$) than control. The genotoxicity of GLY and Roundup Transorb®, a GLY-based formulated, was also reported by Moreno et al. [50] to gill cells and erythrocytes of the fish *P. lineatus*.

Zebrafish (*D. rerio*) has been recommended as a model species of fish toxicity testing, and the use of this organism is increased due to the requirements of replacing/reducing non-mammalian vertebrates in ecotoxicology [51–53]. Zebrafish larvae and adult stages are also used for estimating the genotoxic potential of aquatic contaminants [16,53–55]. However, fish is the most diverse group of vertebrates in aquatic environments and accounts for around 20,000 fish species occupying the aquatic niches [56]. Thus, there is a concern on the reliability of using zebrafish to estimate the hazard of aquatic contaminants to fish in general, due to species differences that can influence the responses to toxicants [57].

In addition, chemicals can trigger different toxic effects depend on the target organ, due to tissue-specific characteristics, such as biochemical and physiological features and xenobiotic metabolic capacity, which can lead to organ-specific genotoxicity [58,59]. To draw a more detail picture of the impact of glyphosate-based formulations and their constituents and breakdown product on aquatic biota, we also investigated the genotoxic potential of GLY, ATN, AMPA e POEA on the permanent fish cell line RTG-2 and observed that only POEA induced

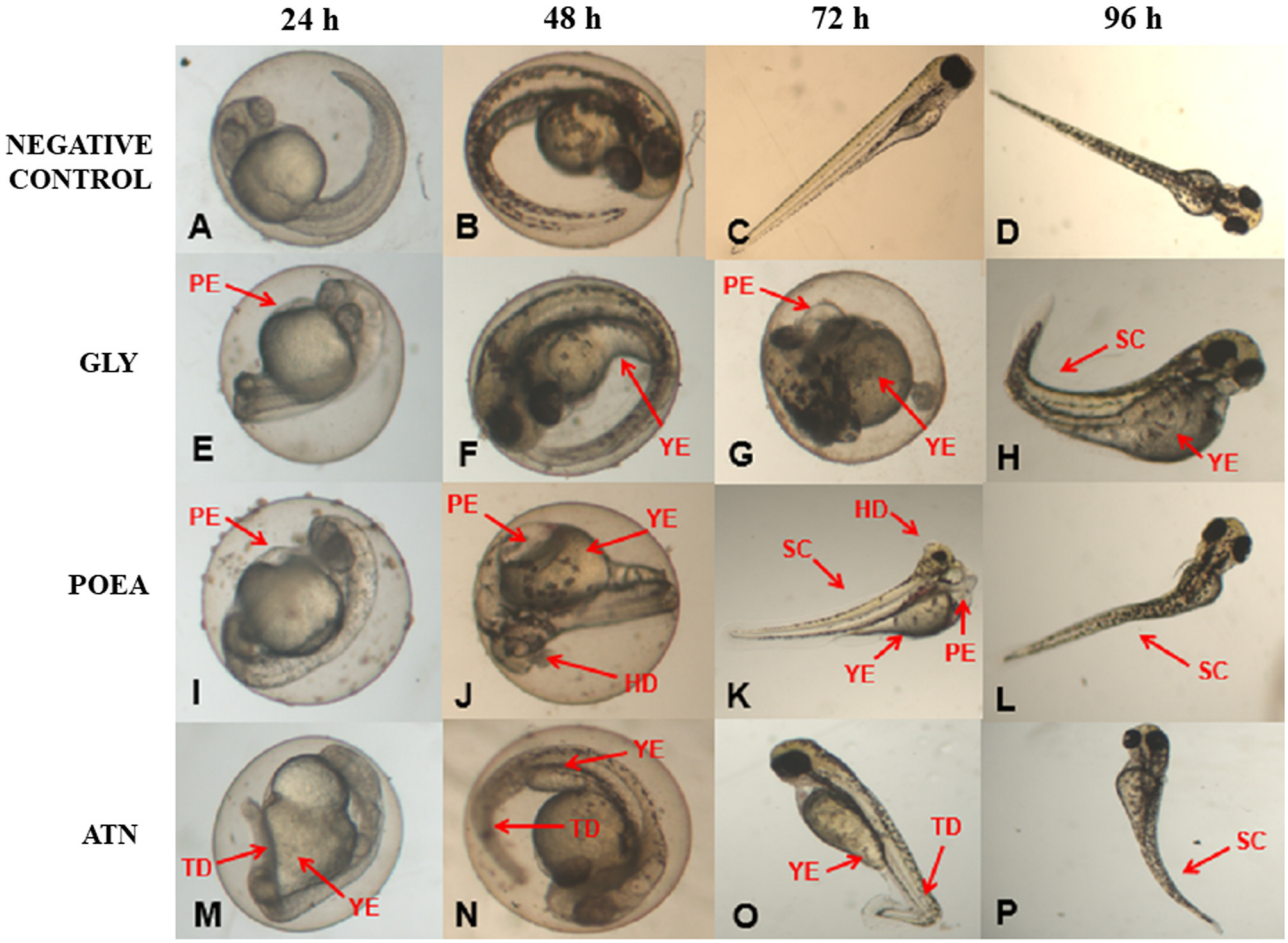

Fig. 2. Zebrafish embryos and larvae abnormalities after GLY, POEA and ATN exposures: pericardial edema (PE), yolk sac edema (YE), spinal curvature (SC), head deformity (HD), tail deformity (TD). Embryos control after 24 h and 48 h of exposure, respectively (A–B); larvae control after 72 h and 96 h of exposure, respectively (C–D), embryos exposed to GLY at 23 mg/L and 100 mg/L for 24 h and 48 h, respectively (E–F); non-hatching embryo exposed to GLY at 10 mg/L for 72 h (G); larvae exposed to GLY at 100 mg/L for 96 h (H); embryos exposed to POEA at 8 mg/L and 4 mg/L for 24 h and 48 h, respectively (I–J); larvae exposed to POEA at 0.4 mg/L and 4 mg/L of POEA for 72 h and 96 h, respectively (K–L); embryos exposed to ATN at 100 mg/L and 50 mg/L for 24 h and 48 h, respectively (M–N); larvae exposed to ATN at 50 mg/L for 72 h and 96 h, respectively (O–P).

DNA damage in this model.

RTG-2 cell line is derived from gonads of the teleost fish rainbow trout (*O. mykiss*), and representing a valid model for genotoxic studies, estimating the impacts of chemicals on the reproductive system of fish [33,36,60–64]. GLY and GLY-based herbicide formulations have also the potential of inducing DNA damages on a variety of fish species as reported by studies conducted with whole organisms instead of single cells in culture [17,57,65]. The uncorrelated genotoxic results between our *in vivo* (whole larvae) and *in vitro* systems can be related to particular physiological features and complexities of these systems, as well as species differences.

Rodrigues et al. [16] correlated the lack of xenobiotic metabolic capability of zebrafish embryos to their low sensitivity of detecting the genotoxicity of the GLY-based herbicides Roundup® and AKB. The capacity of RTG-2 cells in expressing xenobiotic metabolic enzymes has been already demonstrated; however, this cell line presented slow capacity of metabolize benzo(a)pyrene compared to the liver fish cell line PHLC-1, requiring more than 24 h to complete metabolize this promutagen [66]. In our study, zebrafish larvae were exposed to the tested compounds by 96 h whereas RTG-2 cells were exposed by 3 h, which allows the detection of direct genotoxicants.

Therefore, our findings pointed out that POEA may act as a direct genotoxicant while GLY, ATN and AMPA might require metabolic activation to damage DNA. Furthermore, the slow xenobiotic metabolic capacity of gonadal cells can be a “protective” feature against pro-genotoxicants, which lead the reproductive system a non-target organ in exposures to GLY active ingredient and its metabolite AMPA and GLY-based herbicides.

5. Conclusions

In conclusion, these data indicate that the acute effects on zebrafish early-life stages can be ranked in the following order from most to least toxic: surfactant POEA > formulation ATN > active ingredient GLY ≈ metabolite AMPA. Genotoxic effects were observed in both RTG-2 cells (only POEA) and zebrafish (all compounds) with the lowest tested concentrations. It is significant to emphasize that GLY induced DNA damages at an environmentally relevant concentration for zebrafish larvae. Taken together, the present findings confirm that it is important to evaluate different toxicological endpoints as well as use different non-target organisms to predict the hazard of GLY-based formulations and their components and breakdown product to aquatic biota.

Conflict of interest

The authors declare there are no conflicting interests.

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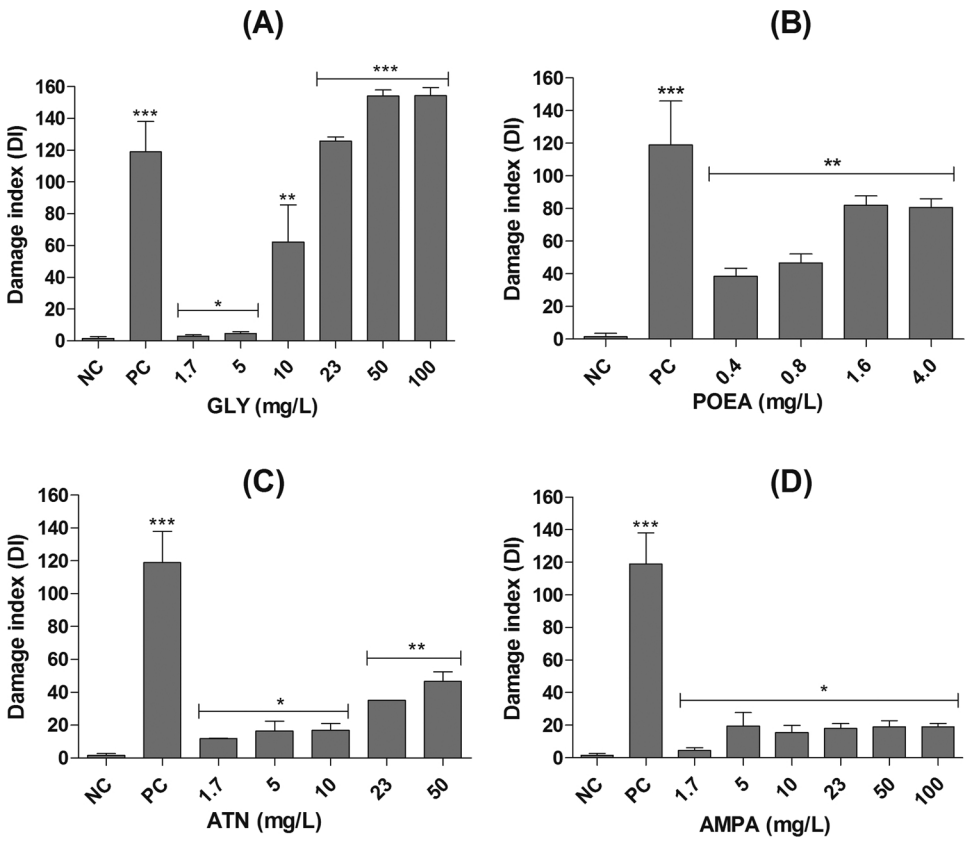

Fig. 3. Damage index (DI) of DNA evaluated by the Comet assay in zebrafish larvae cells after 96 h of exposure to GLY (A), POEA (B), ATN (C) and AMPA (D) using 10–20 surviving larvae per concentration. Bars represent the mean ± standard error of the mean of three independent experiments. *$p < 0.05$, **$p < 0.001$ and ***$p < 0.0001$ statistically different from the respective negative control (NC). PC = positive control (UV light for 10 min at 320 nm).

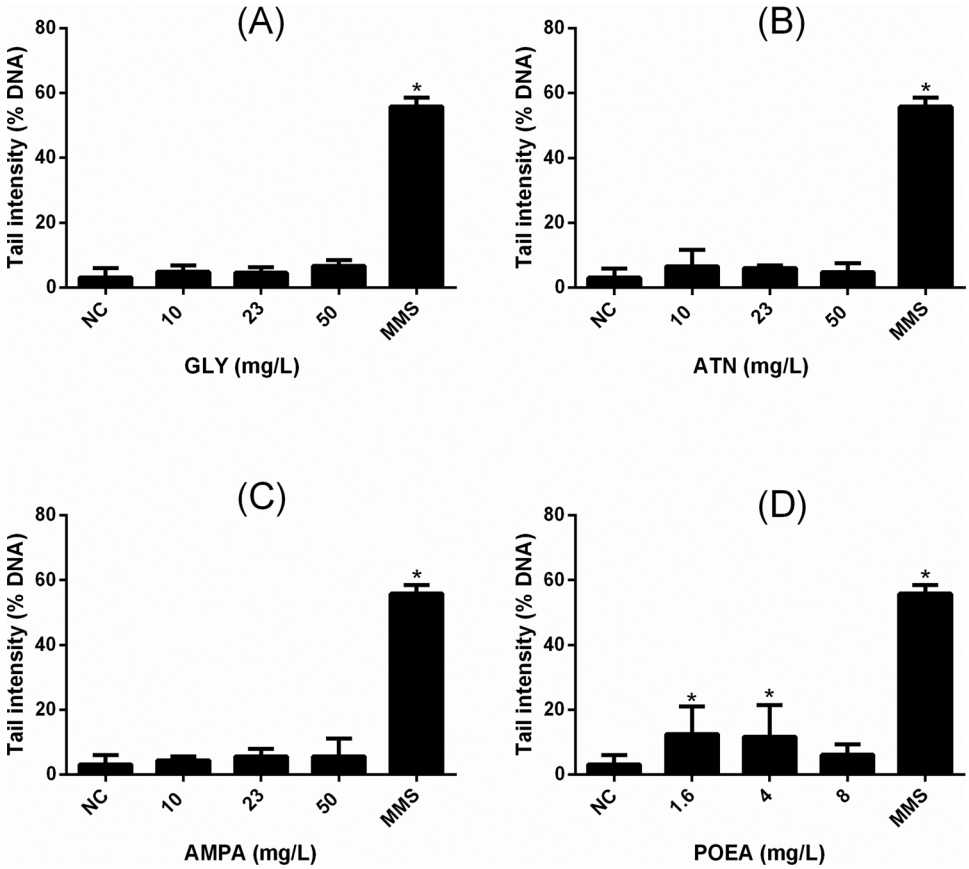

Fig. 4. Genotoxicity evaluation of the GLY (A), ATN (B), AMPA (C) and POEA (D), using the Comet assay with the rainbow trout gonad-2 cell line (RTG-2) after 3 h of exposure. Hundred nucleoids per experimental group were evaluated. Data are expressed as mean ± standard deviation (SD) of % DNA in tail (Tail Intensity) of three independent experiments. NC: negative control; MMS: Methyl methanesulfonate at 0.5 mM after 3 h of exposure as positive control. *$p < 0.05$ statistically different from the respective negative control (NC) based on one-way ANOVA and Dunnett's post hoc test.

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