A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, Oncorhynchus mykiss

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Abstract

Most pesticides used in agriculture end up in the aquatic environment through runoff and leaching of treated crops. One of the most commonly used herbicides is glyphosate. This compound or its metabolites are frequently detected in surface water in Europe. In the present study, *in vivo* and *in vitro* studies were carried out using the early life stages of rainbow trout (*Oncorhynchus mykiss*) and the cell line RTL-W1 (a liver cell line from rainbow trout) to characterize the toxic effects of glyphosate at environmentally-realistic concentrations. Both studies were performed using the commercial formulation Roundup® GT Max, and technical-grade glyphosate for the *in vitro* study. Eyed-stage embryos were exposed for 3-weeks to sub-lethal concentrations (0.1 and 1 mg/L) of glyphosate using Roundup. Numerous toxicity endpoints were recorded such as survival, hatching success, larval biometry, developmental abnormalities, swimming activity, genotoxicity (formamidopyrimidine DNA-glycosylase Fpg-modified comet assay), lipid peroxidation (TBARS), protein carbonyls and gene transcription. Neither concentrations affected embryonic or larval survival, and no significant increases of developmental abnormalities were observed. However, a significant decrease was observed in the head size of larvae exposed to 1 mg/L of glyphosate. In addition, a significant increase in mobility was observed for larvae exposed to the weakest concentration compared to control larvae. Remarkably, TBARS levels were significantly decreased on larvae exposed to 1 mg/L (a.i.), and *cat* and *cox1* genes were differently transcribed from controls. DNA damage was detected by the Fpg-modified comet assay in RTL-W1 cell line exposed to the technical-grade glyphosate and Roundup formulation. The results suggest that sub-chronic exposure to glyphosate, at environmental concentrations, represent a potential risk for aquatic organisms.

Keywords: pesticide, fish embryos, liver cell line, cytotoxicity, embryotoxicity, teratogenicity, genotoxicity, photomotor response

1. Introduction

One of the most commonly used pesticides are the glyphosate-based herbicides, usually transported by agricultural runoff and frequently detected in surface water at high concentrations (Peruzzo et al., 2008). Glyphosate is the active ingredient of Roundup® herbicide; and is commonly used in the form of salt of isopropylamine glyphosate. Glyphosate is a broad-spectrum, non-selective and systemic herbicide for the control of weeds and grass, used in both agricultural and non-agricultural areas. The main degradation products of glyphosate are aminomethyl phosphonic acid (AMPA) and CO_2 (Grandcoin et al., 2017).

Half-life of glyphosate has been determined in several studies, ranging from few days to 2 weeks in freshwater (Giesy et al., 2000). However, its dissipation depends on the local conditions regulated by chemical, physical and biological factors (Giesy et al., 2000), where half-life could last sometimes more than 60 days (Myers et al., 2016).

Environmental exposure to glyphosate is extensive, due to the vast quantities used annually all over the world (Van Bruggen et al., 2018). Increased use of glyphosate is closely linked to the endorsement of genetically modified glyphosate-resistant crops (Van Bruggen et al., 2018), cultivated at about 100 million hectares in 22 countries (mostly soybean, maize, canola and cotton) (http://www.fao.org/docrep/015/i2490e/i2490e04d.pdf). This is particularly true in North and South America, where elevated glyphosate concentrations were reported in different streams and lakes near agricultural basins. For instance, in the Pampa region (Argentina) glyphosate residues were detected up to 4.52 µg/L in surface water (Castro Berman et al., 2018). However, higher concentrations were detected in streams near transgenic soybean cultivation in Pergamino-Arrecifes (North of Buenos Aires), where levels of glyphosate in water varied from 100 to 700 µg/L (Peruzzo et al., 2008). Coupe et al., (2012) studied the fate of glyphosate in different agricultural basins in North America and France, and maximum concentrations of glyphosate were observed between 73 and 430 µg/L.

Several studies have documented the toxicity of glyphosate in various aquatic invertebrates, and acute toxicity thresholds in fish are generally much higher than the concentrations found in streams following applications of crops (Folmar et al., 1979). Commercial formulations of glyphosate seems to be more toxic than the pure molecule, due to interference from substances such as polyethoxilene amine surfactant (POEA) (Folmar et al., 1979; Giesy et al., 2000; Navarro and Martinez, 2014; Tsui and Chu, 2003) which helps the active ingredient penetrate the plant surface. Since fish are susceptible to glyphosate exposure by direct uptake through their gills and via their diet (Giesy et al., 2000), there are several studies that have demonstrated sublethal effects of glyphosate on fish. For example, effects on genotoxicity (Çavaş and Könen, 2007; Guilherme et al., 2012, 2010), acetylcholinesterase (AChE) inhibition (Salbego et al., 2010) swimming alterations (Bridi et al., 2017; Valéria D.G. Sinhorin et al., 2014), reproduction (Uren Webster et al., 2014), and formation of reactive oxygen species (de Moura et al., 2017; Glusczak et al., 2007; Harayashiki et al., 2013; Üner et al., 2006) have been observed in different fish species.

The use of rainbow trout fish (*Oncorhynchus mykiss*) in ecotoxicology is very well documented; and a number of previous studies have looked at the toxicity of glyphosate in this species (Hildebrand et al., 1982; Morgan and Kiceniuk, 1992; Tierney et al., 2007; Topal et al., 2015). Studies have been performed using early life stages (ELS) of fish on the deleterious effects of glyphosate (Sulukan et al., 2017; Yusof et al., 2014; Zebral et al., 2017; Zhang et al., 2017); however, few studies have been done on ELS of rainbow trout. ELS of rainbow trout can be easily raised under laboratory conditions, and because of its slow embryo-larval development, toxicity tests allow longer sub-chronic exposure to toxicants.

On the other hand, the use of cell lines allows the screening of molecules, the study of the mode of action of chemicals and the toxicity assessment of complex environmental samples (Bols et al., 2005; Castaño et al., 2003). Several studies have been done studying the effects of glyphosate on fish cell lines (Alvarez-Moya et al., 2014; Lopes et al., 2018; Qin et al., 2017). For this work, a reference cell line of rainbow trout, RTL-W1 (Rainbow Trout Liver-Waterloo

1), was selected. This cell line, developed by Lee et al. (1993), is derived from untransformed liver tissue of rainbow trout. The RTL-W1 line consists of adherent fibroblastic cells and has the ability to metabolize xenobiotics.

The aim of this work was to study the effects and mechanisms of glyphosate toxicity, using a commercially available product called Roundup on rainbow trout, focusing on ELS, cell cultures and a wide range of endpoints. Exposure of rainbow trout embryos and larvae was conducted using Roundup® for 3 weeks. Several endpoints were studied, such as viability, hatching success, biometric changes, locomotion, genotoxicity, lipid and protein oxidation, and gene transcription. In order to explain some of the observed effects, 10 genes were selected according to their function in antioxidant defense (*cat* and *sod*), detoxification (*gst*), mitochondrial metabolism (*cox1* and *12s*), DNA repair (*ogg1* and *rad51*), apoptosis (*bax*) and reproduction (*er-b* and *cyp19a1*) on fish. In addition, cytotoxicity assays on the RTL-W1 cell line were implemented to screen the toxicity of technical grade glyphosate and Roundup®.

2. Materials and methods

2.1. *In vivo* study: rainbow trout

2.1.1. Test chemicals

Preparation of glyphosate solutions was carried out using the commercial formulation of Roundup® GT Max. The active substance is 480 g/L of glyphosate acid, which is equivalent to 588 g/L of potassium salt of glyphosate. Two stock solutions were prepared at 0.1 and 1 g/L of glyphosate (active ingredient a.i.) with osmosis water. From these stock solutions, exposure solutions at 0.1 and 1 mg/L of glyphosate (a.i.) were prepared.

2.1.2. Exposure system

Eyed stage embryos, at 288 °D (degree days), from rainbow trout (*Oncorhynchus mykiss*) were obtained from INRA-PEIMA (Sizun, FR). Rainbow trout embryos were exposed to 0

(control), 0.1 and 1 mg/L of glyphosate (a.i.) in total darkness and with a temperature of 12°C in a climate chamber for 3 weeks. Each studied condition consisted in 3 replicates with 100 embryos in 1 L aquaria. Exposure solutions was prepared in three 5 L tanks of spring water from Laqueuille (4.7 mg/L Ca, 1.8 mg/L Mg, 5.9 mg/L Na, 2.8 mg/L K, 40.3 mg/L HCO_3^-, 0.2 mg/L SO_4^{2-}, 0.5 mg/L NO_3^-, pH 7.5, <1.2 mg/L Cl^-) and was renewed every two days. A peristaltic pump (Watson Marlow, USA) was used to maintain a continuous flow rate of water (9 mL/min) into the incubation aquaria. Dissolved oxygen concentration was measured each day with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburf, Germany) and data was recorded with OxyView v6.02 software (PreSens Precision Sensor).

2.1.3. Chemical analysis in water

Concentrations of glyphosate and its main metabolite, amino methyl phosphonic acid (AMPA), were analyzed in water samples. Water samples were collected at T_0 and T_{48} (48 h after exposure and before water was changed). Glyphosate and AMPA were measured by the method described by Fauvelle et al. (2015). Briefly, 5 mL of each samples were spiked with 150 µL of glyphosate and AMPA 13C 15N at 20 ng mL^{-1}. Then, 325 µL of 50 mM borate-Na solution and 200 µL EDTA-Na_2 200 mM were added. After homogenization, solutions were left for 5 minutes, 4.5 mL of acetonitrile and 600 µL of FMOC-Cl (50 mg mL^{-1}) were added and samples were left in dark for 30 minutes in order to form FMOC derivates. Acetonitrile was evaporated under nitrogen flow until the volume was below 5 mL. Then, a liquid-liquid extraction with 1.5 mL of ethyl acetate was performed three times. Ethyl acetate was evaporated under nitrogen flow for 15 minutes. One hundred µL of formic acid was added and the sample volume was adjusted to 5 mL. A solid phase extraction was then performed on OASIS HLB cartridges (3 mL, 60 mg, 30 µm particle size, Waters) conditioned with 1 mL of MeOH and 1 mL of formic acid 0.1 %. Samples were loaded on cartridges, and the cartridges were rinsed with 1 mL of formic acid 0.1 % and 1 mL deionized water. The cartridges were

142 dried under nitrogen flow before elution with 2 mL of ammonium hydroxide/deionized
143 water/MeOH 2/30/68. Extracts were evaporated under nitrogen flow until stabilization volume
144 (0.5 mL). The volume was adjusted to 1 mL with deionized water. Analyses was performed by
145 HPLC-ESI MSMS.

146

147 **2.1.4. Embryo-toxicity assay**

148 The viability of embryos and larvae was recorded daily and dead specimens were removed
149 immediately. Half-hatched embryos were considered when part of the body was inside the
150 chorion. Embryonic or larval mortality was the number of dead individuals compared to the
151 total number of embryos at the start of the experiment or total number of hatching larvae. The
152 half-hatched embryo rate was calculated by dividing the number of half-hatched embryos by
153 the total number of embryos at the beginning of the experiment. Hatching time expressed in
154 degree days (DD) was the duration of embryonic development from fertilization to hatching. At
155 the end of the experiment, yolk-sac larvae (540 °D) were placed in Petri dishes with carbonated
156 water and ice to sedate them, and photos were taken for each larva with a stereomicroscope
157 (MZ 7.5 Leica) coupled to a camera CCD (DFP420C Leica) and a cold light (Intralux® 4100,
158 Volpi AG, Schlieren, Switzerland). From the photos, total body length (from the end of upper
159 jaw to the base of the caudal fin) and head length (from the end of the upper jaw to the end of
160 the pectoral fin attachment level) were measured for each larva. The presence of
161 developmental anomalies - including edemas, yolk-sac absorption, spinal malformations,
162 craniofacial anomalies, presence of hemorrhages - was recorded in 15 larvae per replicate
163 randomly chosen.

164

165 **2.1.5. Swimming behavior analysis**

Analysis of swimming behaviour was carried out on 12 yolk-sac larvae per replicate at 528 DD. The larvae were acclimated individually 30 minutes in the dark at 12°C in 6-well microplates containing 8 mL of exposure solution. The microplates were placed in the recording chamber (Daniovision Image Analysis System with Ethovision software version 12.0 Noldus) connected to a thermoregulation system set at 12 ± 0.5°C (Pilot one®, Huber). Larvae were subjected to a light/dark cycle of 30 minutes, divided into 10 minutes dark, 10 minutes light, and 10 minutes dark. This cycle is designed to analyze the photomotor response of larvae in response to light stimulation. An infrared camera in the recording chamber records the movement of each larva focusing on their center of gravity. The average velocity of each larva was calculated over 30 seconds. The total distance traveled, time of mobility and the time spent in the peripheral area of the wells were determined for each larva.

2.1.6. Biochemical analyses

Preparation of supernatant

At the end of the exposure, 4 pools of 2 yolk-sac larvae were frozen in liquid nitrogen and stored at -80°C until analysis. Larvae (approximately 250 mg) were homogenized in a phosphate buffer (0.1 M; pH 7.5; 4°C) using an UltraTurrax® tissue homogenizer fitted with a potter at 3,000 rpm (4°C). Then, samples were centrifuged at 9,000 g for 25 min at 4°C. The supernatant S9 fraction obtained were placed in different tubes for total protein, TBARS and protein carbonyl measurements.

Total protein

The total protein concentration was determined using the method of Lowry et al. (1951) on S9 fraction. Bovine Serum Albumin (BSA) was used as a standard. Measurements were performed using a spectrophotometer microplate reader (Synergy HT, BioTek).

Lipid peroxidation (TBARS)

Lipid peroxidation was assessed following the method of Buege and Aust (1978) adapted to a microplate reader. Five hundred µL of S9 fraction were added to 500 µL of a solution containing 20 % of butylated hydroxytoluene (BHT) and 20 % of trichloroacetic acid (TCA). The mixture was then centrifuged for 10 min at 9,000 g. Afterwards, 600 µL of supernatant was added to 480 µL of TRISbase (25 mM) - TBA (thiobarbituric acid – 100 mM) and 120 µL of 0.6N HCl and heated at 80°C for 15 min. Mixtures were subsequently cooled and mixed. TBARS levels were read using a UV-spectrophotometer (Synergy HT, BioTek) in a microplate at 530 nm. Results were expressed as nmoles of thiobarbituric acid reactive substance (TBARS) equivalents/mg of protein.

Carbonylated protein analysis

Carbonylated protein content was measured using the method described in Augustyniak et al. (2015). 50 µL of 11 % streptomycin sulfate – phosphate buffer (100 mM pH 7.4) was added to 500 µL of S9 fraction, mixed and incubated for 15 min at room temperature. Then, the mixture was centrifuged for 10 min at 6,000 g. Afterwards, supernatant was divided into two tubes (200 µl each) where 200 µL of supernatant was added to 800 µL of HCl 2.5 M used as a control tube, and 200 µL of supernatant was added to 800 µL of DNPH (2.4-dinitrophenylhydrazine 10 Mm) used as a sample tube. Subsequently, the mixture was incubated for 1 h at room temperature with vortexing every 15 min. Proteins were precipitated with 1 mL of 20 % TCA (trichloroacetic acid), vortexed and centrifuged for 10 min at 10,000 g. The pellets were rinsed with 1 mL of ethanol-ethyl acetate (v:v), vortexed and centrifuged three times. Pellets were then solubilized with 500 µL of 6 M guanidine HCl and centrifuged at 10,000 g for 10 min. The

carbonyl content was measured using a UV-spectrophotometer (Biotek Synergy HT) at 370 nm. Results were expressed as nmoles of DNPH incorporated/mg protein.

2.1.7. Gene expression

Six yolk-sac larvae per replicate were collected individually in a storage buffer (RNA later, Qiagen). Samples were deep-frozen in liquid nitrogen and then stored at -80°C until analysis.

RNA extraction

Total RNA extraction from whole larvae was done following the kit “SV Total RNA Isolation system” (Promega) according to the supplier’s recommendations. This kit included a DNaseI treatment to avoid genomic DNA contamination of the samples. All details of RNA extraction are described in Weeks et al. (2019). For each exposure condition, samples were analyzed in triplicate.

Retro-transcription of total RNA into cDNA

The retro-transcription of total purified RNA was realized with the kit “GoScript Reverse Transcription System” (Promega), following the indications described at Weeks et al. (2019). The cDNA thus obtained were stored at -20°C pending analysis by quantitative real-time PCR reaction.

Quantitative real-time PCR

Twelve genes were selected and specific primer-pairs were designed with primer3plus software (Table 1). All primer-pairs used in this study has an efficiency upper than 95 %. Real-time qPCR was carried out using GoTaq® qPCR Master Mix kit (Promega) and was performed in a Mx3000P® qPCR System (Stratagene), as fully described in Weeks et al. (2019). For each reaction, specificity of amplifications was determined from the dissociation curve of the PCR

products. This dissociation curve was obtained by following the SYBR Green fluorescence level during a gradual heating of the PCR products from 60 to 95 °C.

Cycle thresholds (Ct) were obtained from MxPro™ qPCR software for each gene. Two different housekeeping genes were used for standardization (*rpl7* and *ef1α*) and were found to be stable in our conditions. Consequently, relative quantification of each gene expression level was normalized according to the mean Ct value of these two reference genes and using the 2ΔCt methods (Livak and Schmittgen, 2001). The expression factor (induction if >2 and repression if <0.5) of each gene was calculated for each condition by dividing the transcription level of exposed individuals by that observed in control ones

2.2. *In vitro* study using RTL-W1 cell line

2.2.1. Cell exposure

The RTL-W1 cell line was obtained from rainbow trout liver (Lee et al., 1993). For cell culture, L15 Leibovitz medium supplemented with 5 % FBS (Fetal Bovine Serum) and 1 % Penicillin/Streptomycin (100 IU/mL) was used. The cells were kept in polypropylene flasks of 75 cm^2 (Cell start® cell culture Flask Greiner) at 20 °C. The analysis was carried out with cells aged from passage from 65-72.

The cytotoxicity and genotoxicity test were carried out in 96- and 24-well polypropylene microplates, respectively. For both MTT and comet assay, cell lines were seeded 24 h prior glyphosate exposure in triplicate. Cell density was 200 000 cells/mL. For the MTT assay, cell lines were exposed to concentrations from 0.05 to 1000 mg/L of glyphosate for 24 h, using both technical and commercial formulation Roundup®. For the comet assay, the concentrations tested were the same studied in our *in vivo* study (0.1 and 1mg/L of glyphosate) using technical and commercial formulation of glyphosate.

2.2.2. MTT assay for cytotoxicity evaluation

The cytotoxicity test was performed using serum free L15 medium containing 10 % of 3(4,5-dimethyl-2thiazholyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT). 24 h after chemical exposure, the medium was removed and cells were rinsed with PBS. 100 µL of the MTT solution was added to the wells. After incubation of 1 h in the dark (time to allow cells to reduce tetrazolium to formazan), the MTT solution was removed and 100 µL of isopropanol solution (4% 1N HCl) was added. Then the microplate was shaken horizontally in the dark for 15 min to dissolve the formazan crystals. Following this step, the formazan coloration was quantified in a Bio-Tek Synergy HT spectrophotometer at 570 nm.

2.3. Genotoxicity test

The alkaline comet assay was performed following Le Bihanic et al. (2014) and Weeks-Santos et al. (2019) in blood cells of larvae, and RTL-W1 cell line following Pannetier et al., (2018). Blood sampling was performed in 6 larvae per replicate (previously anesthetized with ice water and few drops of carbonated water) by decapitation using a heparinized pipette. Samples were stored in microtubes with 200 µL of cryopreservation solution (250 mM sucrose, 40 mM citrate trisodique, 5 % DMSO, pH adjusted to 7.6 with nitric acid 1 M) and immediately frozen in liquid nitrogen until analysis. The comet assay for the RTL-W1 cell line (1.5 to 2 x 10^5 cells/mL) was performed 24 h after cell exposure to glyphosate. For each condition, 4 replicates were prepared. The cells were rinsed, trypsinized and transferred into microtubes. The cells were then centrifuged (5 min, 20 °C at 1000 rpm) and supernatant was removed. Cell pellets were re-suspended in 100 µL of L15 medium (without FBS) before being mixed with 200 µL of low melting point agarose (0.75 % LMPA). A 50 µL of cell suspension (blood and cell line) were deposited on slides previously coated with NMPA (Normal Melting Point Agarose, 0.8% w/w) and covered with an 18x18 mm coverslip. The slides were then immersed in a lysis solution (10 mM Tris; 2.5 M NaCl; 100 mM EDTA; 1% Triton X-100; 10% DMSO; pH adjusted to 10 with NaOH) at 4°C for 90 min in the dark. At the end of the lysis, the slides were then rinsed 3 times for 5 min in an enzyme buffer at pH 8 (Biolabs, Evry, France). Then the slides were immersed for 30 min in two hellendahls, the first one containing 60 mL of buffer with 12 µL of

289 the enzyme Fpg (Biolabs, Evry, France) diluted in 1/5000, and the second one with only buffer.
290 Following exposure to enzymes, the slides were incubated in an alkaline solution (0.3 M NaOH,
291 1 mM EDTA, pH> 13) at 4°C for 40 min for the RTL-W1 cells and 20 min for blood cells to
292 allow the DNA to unwind. Electrophoresis was then performed in the same solution at a voltage
293 of 25 V and 300 mA for 20 min. The slides were rinsed 3 times with neutralizing solution (0.4
294 M Tris, pH 7.5) for 5 min at 4°C. Afterwards, the slides were dehydrated in absolute ethanol
295 for 20 min and then allowed to dry at room temperature for at least 12 h. Slides were stained
296 with 20 µg/mL of ethidium bromide solution and covered with a 22x22 mm coverslip. Comet
297 analysis was carried out using an epifluorescence microscope (Olympus BX51) (zoom x20)
298 equipped with an Olympus U-RFL-T reflected fluorescence system lamp. The comets were
299 quantified using the Comet Assay IV software (Instrument Perspective LtD). Results are
300 expressed as percentage of degradation of DNA tail for 100 randomly selected nuclei per slide.

301

302 **2.4. Statistics**

303 Sampling of larvae (individuals and pools), from each exposure condition, were performed in
304 triplicate and each replicate was considered as an independent sample. All data are expressed
305 by the mean ± SE (Standard Error). For the MTT test, the EC_{50} was calculated by PRISM 5
306 software (GraphPad software, California, USA). Statistical analyzes were carried out using R
307 (http://cran.r-projet.org/). The Normality of data distribution was verified on the residues by the
308 Shapiro-Wilk test ($p < 0.01$) and the homogeneity of variances was evaluated by the Levene
309 test ($p < 0.05$). In the case of normal distribution, a one-way ANOVA analysis was used
310 ($p < 0.05$) followed by a Tukey post-hoc test. In the case that data was not normal,
311 comparisons were carried out by non-parametric tests of Kruskal-Wallis ($p < 0.05$).

312

313 **3. Results**

314 **3.1. Exposure conditions**

315 Table 2 shows the concentrations of glyphosate in water for each experimental conditions. The
316 analyses were carried out at 0 and 48 hours after exposure to estimate the possible losses of
317 the molecule. The measured concentrations of glyphosate were comparable (± 20 %) to the
318 nominal concentrations. No concentration variation was noted during 48h. The glyphosate's
319 metabolites, aminomethylphosphonic acid (AMPA) were also analyzed but not detected at T0
320 and T48.

321 **3.2. Embryonic and larval survival**

322 Dissolved oxygen in the exposure water varied between 83.8 and 93% throughout the duration
323 of this study. Exposure to 0.1 and 1 mg/L of glyphosate (a.i.) did not induce significant mortality
324 in trout embryos and larvae throughout the duration of exposure (table 3). Both, embryonic
325 and larval survival, were greater than 90 % in all studied conditions. All embryos hatched
326 successfully. The duration of development was slightly longer for both groups exposed to
327 glyphosate compared to control, however no significant differences were observed.

328 **3.3. Biometry**

329 No significant differences were observed in total larval length between the studied conditions
330 and the control (Figure 1-A). Nevertheless, measurement of larvae head size showed
331 significant decreases in larvae exposed to 1 mg/L of glyphosate compared to control (Figure
332 1-B). Head size in unexposed larvae was 4.76 ± 0.04 mm against 4.55 ± 0.11 mm for larvae
333 exposed to 0.1 mg/L of glyphosate, and 4.43 ± 0.14 mm on larvae exposed to
334 1 mg/L of glyphosate. The ratio between total length and head size (Figure 1-C) showed a
335 significant dose-dependent decrease from control (24.79 ± 0.14 %) and larvae exposed to
336 0.1 mg/L of glyphosate (24.14 ± 0.27 %) and 1 mg/L of glyphosate (23.37 ± 0.3%).

337 **3.4. Malformations**

338 Embryo–larval exposure to glyphosate did not result in significant induction of malformation
339 when compared to non-exposed larvae. Control condition presented 13.3 ± 6.7 % of

malformed larvae. However, larvae exposed to 1 mg/L of glyphosate (a.i.) showed a significant increase in developmental anomalies over larvae exposed to 0.1 mg/L of glyphosate (a.i.) with 26.7 ± 6.7 % and 8.9 ± 3.8 % respectively (Figure 2).

3.5. Swimming behavior

Figure 3 (A and B) shows the responses of larvae to light stimulation. Results represent the average speed of larvae exposed to glyphosate with alternating periods of luminosity. Under each condition, the same tendency was observed with an increase in larval velocity during the light period. No significant differences were observed at the first period of darkness when comparing the different treatments (Figure 3-A and B). When the light was turn on, the stress caused an increase in the average speed of the larvae exposed to 0.1 mg/L of glyphosate with a pic of 29.2 ± 2.3 cm/s when compared to control and larvae exposed to 1 mg/L of glyphosate (22.4±1.5 and 23.1±3.1cm/s respectively) (Figure 3-B). However, after 4 min of light exposure, this increase of velocity was no longer different for larvae exposed to 0.1 mg/L of glyphosate when compared to other conditions (Figure 3-A). Likewise, no significant differences were observed at the second dark period.

Figure 4 shows the average cumulative time of immobility, mobility and high mobility for larvae exposed to both glyphosate conditions and control. Larvae exposed to 0.1 mg/L of glyphosate were significantly highly mobile (8.04 ± 1.25 s) in the light period when it was compared to control (4.72 ± 0.63 s) and larvae exposed to 1 mg/L of glyphosate (4.19 ± 0.38 s).

3.6. Genotoxicity in blood cells

The average level of DNA damage for each studied condition, with and without treatment by Fpg is presented in Figure 5. No significant differences were observed in DNA damage in all conditions when cells were not treated with Fpg enzyme (6.85 ± 2.11 % for control, 8.52 ± 2.33 % for 0.1 mg/L of glyphosate condition, and 7.28 ± 1.69 % for 1 mg/L of glyphosate condition). A global increase of DNA damage was observed after Fpg treatment but no

365 significant differences were observed between conditions (20.86 ± 3.73 % for control
366 condition, 22.37 ± 2.12 % for larvae exposed to 0.1 mg/L of glyphosate and 19.88 ± 1.02 % for
367 larvae exposed to 1 mg/L of glyphosate).

368

369

370 **3.7. Lipid peroxidation (TBARS) and protein carbonyls**

371 TBARS levels showed a significant reduction in larvae exposed to 0.1mg/L of glyphosate when
372 compared to control (figure 6-A). In the other hand, larvae exposed to glyphosate did not show
373 any significant changes in protein carbonyls (figure 6-B).

374 **3.8. Gene expression**

375 After 3-week exposure of rainbow trout to glyphosate, only a handful of significant changes
376 were observed on gene expression on larvae exposed to 1 mg/L. *Cox1* gene was significantly
377 down-regulated (0.22) when *cat* gene level was increased (2.13). The expression of *sod*, *gst*,
378 *ERb*, *12s*, *ogg1*, *rad51*, *bax* and *Arom* were not significantly differentially regulated following
379 glyphosate exposure (data not showed).

380 **3.9. Cytotoxicity**

381 The cytotoxicity data for glyphosate and Roundup® (a.i.) was obtained using the MTT assay
382 on RTL-W1 (Figure 7). Cytotoxicity was observed only at concentrations above 250 mg/L of
383 glyphosate, and 200 mg/L of Roundup® (a.i.). The EC_{50} calculated at 24 h was 730 and
384 710 mg/L for glyphosate and Roundup® (a.i.), respectively.

385 **3.10. Genotoxicity in RTL-W1 cell line**

386 With the standard comet assay, no genotoxic effect was detected after exposure to both
387 glyphosate and Roundup® whatever the tested concentrations. However, with the modified

388 Fpg assay, significant genotoxic were observed on RTL-W1 cell line exposed to 0.1 and 1 mg/L
389 of technical glyphosate with 33.6±3.1 and 33.5±3.2% of DNA damage, respectively, when
390 compared to control condition with 25.4 ± 2.9 % of DNA damage (Figure 8). The same was
391 observed using Roundup® formulation where significant DNA damage was at 26.8 ± 1.5 and
392 23.9 ± 2.3 % for cells exposed to 0.1 and 1 mg/L of Roundup® (a.i.) when compared to control
393 with 17.9 ± 2.1 % of DNA damage (Figure 8).

394

395 **4. Discussion**

396 According to the World Health Organization (WHO, 1996), the acute toxicity of Roundup is
397 considered to be low in vertebrates. Because of its widespread use, and its slow degradation,
398 this herbicide is often found in aquatic environments at relatively high concentrations (Vera et
399 al., 2010) and thus could represent a threat for pollutant-sensitive species or early life stages
400 (ELS). Several authors have studied the acute toxicity of glyphosate on ELS, fingerlings and
401 adults of rainbow trout (Folmar et al., 1979; Hildebrand et al.,1982; Morgan and Kiceniuk, 1992;
402 Anton et al., 1994). 96h LC_{50} for rainbow trout embryos and larvae was estimated to 16 and
403 3.4 mg/L glyphosate (a.i.) respectively (Folmar et al., 1979). However, acute toxicity varies
404 according to the commercial formulation. For example, 96 h LC_{50} on rainbow trout fingerlings
405 was estimated to be 54.8 mg/L using Roundup® formulation (Hildebrand et al., 1982); and
406 10.4 mg/L using Vision formulation (Morgan and Kiceniuk, 1992). ~~The work of Yusof et al.~~
407 ~~(2014) focused on glyphosate toxicity on Java medaka. Their results showed that 50 % of~~
408 ~~embryos exposed to 100 mg/L of glyphosate died after 16 days of exposure, and a decrease~~
409 ~~on hatching rate in a concentration-dependent manner from 100 to 500 mg/L of glyphosate.~~

410 The *in vitro* study analyzed the toxicity of glyphosate using the rainbow trout liver cell line (RTL-
411 W1) considering technical grade glyphosate and its commercial formulation Roundup. The
412 toxicity test carried out on trout liver cells may provide additional information about the toxicity
413 mechanistic of pollutants (Bols et al., 2005; Castaño et al., 2003). The RTL-W1 cell line can

414 be considered a suitable model, given that the liver is the main organ responsible for
415 metabolising pollutants (Belpaeme et al., 1998). The results obtained on RTL-W1 in this study
416 highlight the cytotoxic effects of glyphosate, but at high concentrations above 200 mg/L. Our
417 results also indicate that the commercial formulation is slightly more cytotoxic than the
418 technical grade compound, which could be related to the presence of additives, especially
419 surfactants (POEA) in the commercial formulation. Similar studies on human cell lines have
420 shown that glyphosate-based formulations are usually more cytotoxic that the technical grade
421 compound (Gasnier et al., 2009; Koller et al., 2012; Martínez et al., 2007; Mesnage et al., 2013;
422 Vanlaeys et al., 2018). In addition, the study of Gasnier et al. (2009) evidenced that the
423 concentration of glyphosate in the commercial formulation is not related to toxicity. Indeed, the
424 formulation containing 400 g/L of glyphosate (a.i.) (Grands Travaux®, homologation 8800425)
425 has a lower LC_{50} than its homolog containing 450 g/L (Grands Travaux plus®, homologation
426 2020448) confirming that the nature and concentration of adjuvants have a real impact on the
427 toxicity of the mixture. Very few studies have been done on fish cell lines regarding the toxic
428 effects of glyphosate. The LC_{50} of glyphosate on diploid and triploid fin cell lines from
429 *Misgurnus anguillicaudatus* (DIMF and TRMF) were 315.34 and 371.77 mg/L respectively (Qin
430 et al., 2017). Cytotoxicity of Roundup was also studied on zebrafish cell line ZF-L regarding
431 the integrity of the plasma membrane, mitochondrial activity and lysosomal integrity. The
432 authors reported a significant reduction of cell viability from 67.7 µg/L (a.i.) (Goulart et al.,
433 2015). LC_{50} of mononuclear blood cells was determined at 56.4 mg/L for Roundup, and
434 1630 mg/L for technical grade glyphosate (Martínez et al., 2007). These differences of toxicity
435 might depend on the concentration of the active agent but also the nature and concentration
436 of its adjuvants, as well as the cell line used.

437 In the literature, there are few studies concerning the effects of glyphosate on fish growth and
438 the findings are often inconsistent. Rainbow trout fingerlings exposed up to 100 µg/L of
439 glyphosate (a.i.) using Vision formulation (Monsanto Co.) did not show significant effect on
440 length or weight after two months of exposure (Morgan and Kiceniuk, 1992). *Leporinus*

441 *obstusidens*, a South American fish species, was exposed to 1 and 5 mg/L of glyphosate (a.i.)
442 using Roundup for 90 days and exhibited a lower growth rate (with reductions between 10 and
443 15 % respectively) and a lower weight gain (between 44 and 65 % respectively) when
444 compared to control fish (Salbego et al., 2010). Similarly, Bridi et al. (2017) reported a reduced
445 body length in zebra fish larvae (*Danio rerio*) exposed from 0.01 to 0.5 mg/L of Roundup (a.i.)
446 for 96 h. Koakoski et al., (2014) also observed a reduction of the weight gain and biomass of
447 *Rhamdia quelen* fingerlings when exposed to 1.21 mg/L of Roundup for 96 h and after 180
448 days of depuration. ~~Another study using adult fishes (*Piaractus Mesopotamicus*) reported that~~
449 ~~glyphosate reduced food intake and therefore could have an impact on normal growth~~
450 ~~(Cardoso Giaquinto et al., 2017).~~ Furthermore, some authors have stated that glyphosate may
451 have an effect on growth hormones and cortisol levels in fish (Cericato et al., 2008; El-Shebly
452 and El-kady, 2008; Koakoski et al., 2014). Cericato et al. (2008) observed that cortisol levels
453 in fish exposed to glyphosate were higher than in non-exposed fish. Indeed, cortisol is released
454 in response to stress and contributes to restore homeostasis (De Boeck et al., 2001), and
455 some evidence suggest that elevation of cortisol might interfere with normal growth of fishes
456 by stimulating energy-consuming processes (Bernier et al., 2004; De Boeck et al., 2001). In
457 our study, a 3-week exposure of rainbow trout embryos to 0.1 and 1 mg/L glyphosate did not
458 induce significant reductions in total body length of larvae. However, head length of larvae was
459 significantly smaller for those exposed to the highest tested concentration, and the ratio of
460 head to total body length showed a significant decrease in a concentration-dependent manner.
461 Interestingly, Zebral et al. (2017) evaluated eye diameter and distance between eyes in
462 pejerrey embryos (*Odontesthes humensis*) exposed to this herbicide (0.36-5.43 mg/L) for 96 h
463 and observed that both parameters were significantly reduced in a concentration-dependent
464 manner in exposed groups. Similar results were found by Zhang et al. (2017) in zebra fish
465 embryo (*D. rerio*) but using higher concentrations (up to 400 mg/L) of glyphosate for 96 h.
466 Zebral et al. (2017) suggested that glyphosate might alter the retinoic acid pathway, which
467 plays a major role in growth and development. Paganelli et al. (2010) also indicated that
468 glyphosate produces teratogenic effects on vertebrates by impairing retinoic acid signaling.

~~Our results showed a trend of increasing spinal deformities when rainbow trout embryos were exposed to 1 mg/L of glyphosate. Several studies have reported significant body malformations, spinal curvature, pericardial and yolk sac edemas on embryos of zebra fish (Sulukan et al., 2017; Zhang et al., 2017) and Java medaka (Yusof et al., 2014) using relatively high concentrations of Roundup® from 1 to 500 mg/L (a.i.).~~

Larvae exposed to 0.1 mg/L of Roundup® (a.i.) were more active under light stimulation. Several previous studies have also examined the effects of glyphosate on fish swimming behaviour. In concordance with our results, Morgan et al., (1991) observed that after one-month exposure to 45.75 µg/L of glyphosate, under Vision's commercial formulation, fry rainbow trout presented erratic and agitated behaviour compared to unexposed fish. Similar abnormal behaviours and hyperactivity were also reported in Nile tilapia (Ayoola, 2008) and *Tilapia zillii* (Nwani et al., 2013) exposed from 2 to 310 mg/L for 4 days and from 216 to 540 mg/L of glyphosate for 96 h, respectively. A Neotropical hybrid fry fish, surubim, showed increased swimming activity and ventilation frequency 96 h after exposure to 7.5 and 15 mg/L of Roundup® (a.i.) (Sinhorin et al., 2014). In the other hand, Bridi et al., (2017) observed that zebrafish larvae and adults exhibited significant reduction of distance travelled and mobility when exposed to glyphosate and Roundup® formulations (0.01 to 0.5 mg/L a.i.) for 96 h. The behavioural study of this work was performed in larvae after 21 days of glyphosate exposure. The absence of behavioural changes at the dark period could mean an adaptation of response to stress. It was shown that sub-chronic exposure to low concentrations of glyphosate (0.1 mg/L a.i.) induced an increase in swimming behaviour in exposed rainbow trout larvae but no effect on swimming activity was observed at 1 mg/L. This apparent hyperactivity decreased 4 min later of light exposure. Same patrons were observed by Zhang et al. (2017) where locomotive activities in day time of zebrafish larvae, exposed to low concentrations of glyphosate (0.01 and 0.5 mg/L a.i.) were increased; however, at stronger concentrations (5 mg/L a.i.) this increase was no longer observed when compared to non-exposed larvae.

These alterations may have a consequence in the response face to predators or other danger (Zhang et al., 2017).

In this study, the use Fpg-modified comet assay improved detection threshold for DNA damage. The standard comet assay can detect single or double strand breaks and alkali-labile sites, while the addition of Fpg enzyme can also detect lesions related to alkylation damage, abasic sites (apuric or apyrimidic) and oxidative damage (8-oxoGua) induced by ROS (Reactive Oxygen Species) production (Kienzler et al., 2012). In our exposure conditions, glyphosate did not induce any DNA strand breaks on blood cells of rainbow trout larvae after 21 days of exposure. However, some studies have demonstrated the genotoxic potential of Roundup® in different fish species like *Anguilla anguilla* (Guilherme et al., 2012, 2010), *Corydoras paleatus* (De Castilhos Ghisi and Cestari, 2013), *Prochilodus lineatus* (Moreno et al, 2014) and *Carassius auratus* (Çavaş and Könen, 2007). Guilherme et al., (2010) showed Roundup®'s capacity to induce DNA single strand breaks and cytogenetic effects on blood cells of European eel using low concentrations (58 and 116 µg/L a.i.) after 1 and 3 days of exposure. Cavalcante et al., (2010) observed genotoxic potential of Roundup® on blood and gill cells after 6 h of exposure to 10 mg/L (a.i.) on fish (*Prochilodus lineatus*), but DNA damage returned to the baseline level after 24 and 96 h of exposure for erythrocytes and gill cells respectively. The activation of the antioxidant and DNA repair systems after glyphosate exposure have already been demonstrated by Cavalcante et al., (2010) and Marques et al., (2014). In our case, we may assume ROS were produced but larvae were able to activate protective mechanisms such as DNA repair enzymes to prevent DNA damage on blood cells, as reported in several articles (Marques et al., 2014; Ching et al., 2001; Kienzler et al., 2013).

On the other hand, Fpg-modified comet assay in RTL-W1 cell line indicated that both technical grade glyphosate and Roundup® induced abasic sites and oxidative DNA damage at concentrations of 0.1 and 1 mg/L (a.i.), but no significant increase in DNA damage was observed with the classical comet assay. Observing a genotoxic on RTL-W1 (short exposure), and not on larvae (longer exposure) favours the hypothesis of the activation of *in vivo* repair

systems. However, we must be cautious with this comparison because the studied cells are not the same *in vivo* and *in vitro*. No genotoxicity studies of glyphosate have been performed on RTL-W1 cell line. Using the human hepatoma cell line, HepG2, no DNA damage was observed when glyphosate was tested as a pure form after an exposure of 4 h (Kašuba et al., 2017). In human buccal epithelial cells, TR146, glyphosate and Roundup induced DNA damage from 20 mg/L and DNA damage increased as a function of the exposure concentration (Koller et al., 2012). Differences in genotoxicity activity were observed between *in vitro* and *in vivo* exposure in tilapia erythrocytes after exposure to glyphosate (a.i.) (0.0007 - 0.7 mM) (Alvarez-Moya et al., 2014). *In vitro*, DNA damage was proportional to glyphosate concentration; however, *in vivo*, glyphosate was genotoxic to fish erythrocytes but not in a concentration-dependent manner.

Malondialdehyde (MDA) is one of the secondary products that can be formed during lipid peroxidation of uncontrolled oxidative stress in cells (Ayala et al., 2014). It is considered as the most mutagenic product of lipid peroxidation, and once formed, MDA can react with proteins or DNA to form adducts resulting in biomolecular damage (Ayala et al., 2014). Because of its easy reaction with thiobarbituric acid (TBA), MDA has been used as a convenient biomarker of lipid peroxidation using the thiobarbituric acid reacting substances test (TBARS) (Ayala et al., 2014). Lipid peroxidation (LPO) has already been studied in fish exposed to glyphosate based herbicides and results might be very variable according to fish species, exposure duration (Glusczak et al., 2007; Modesto and Martinez, 2010; Sinhorin et al., 2014), gender (Harayashiki et al., 2013) and tissues (Glusczak et al., 2007; Sinhorin et al., 2014). Juveniles of *Prochilodus lineatus* have significantly increased LPO levels in liver after 6 h of exposure to both 1 and 5 mg/L of Roundup Transorb. However, these alterations returned to control levels after 24 h of exposure (Modesto and Martinez, 2010). On the other hand, Glusczak et al. (2007) did not observed TBARS alterations in liver of silver catfish (*Rhamdia quelen*) when exposed to 0.2 and 0.4 mg/L, but they did in muscle tissue at both concentrations. ~~Ferreira et al. (2010) also studied the oxidative stress of different pesticides in silver catfish finding that methyl~~

~~parathion and tebuconazole but glyphosate enhanced TBARS levels in liver of fish.~~ The hybrid amazon fish surubim had significantly increased TBARS levels in both liver and muscle, but not in the brain after exposure to 2.25 to 15 mg/L of Roundup (Sinhorin et al., 2014). Even though several authors have studied TBARS levels in fish exposed to glyphosate, only few analyses have been done on whole larvae. Our results show that TBARS levels were reduced in whole larvae exposed to 0.1 mg/L of glyphosate when compared to control group. Fish have a natural anti-oxidative defense system against free radicals, and are able to reduce oxidative damage to below control levels (Marques et al., 2014). As hypothesized by Marques et al. (2014), a development of antioxidant systems may occur as a response to ROS, reducing the vulnerability of cells and their constituents. Reduced levels of lipid peroxidation have already been observed in the livers of male guppy exposed to 700 µg/L of Roundup (a.i.) (Harayashiki et al., 2013), in brain of piava fish (*Leporinus obtusidens*) exposed from 3 to 20 mg/L of glyphosate commercial formulation (a.i.) (Glusczak et al., 2011). Lipid peroxidation may not only depend on ROS production, but may be also be affected by physiological transitions that occur at different developmental stages (Cao et al., 2010; Mourente et al., 1999). The presence of carbonyl groups in proteins induced by glyphosate was also studied in several reports (de Moura et al., 2017; Glusczak et al., 2011; Sinhorin et al., 2014) generally in liver since it is consider as the main site of protein carbonyl production (Sinhorin et al., 2014). In contrast, the absence of protein carbonyl changes in our results could also indicate, once again, that the antioxidant system of rainbow trout larvae functions efficiently to defend against oxidative stress. As for TBARS, only a few analyses have been done using whole fish larvae to analyze carbonyl groups in proteins. Considering that protein carbonyl formation is non-reversible (Zhang et al., 2008), it can be suggested that at this developmental stage of larvae, ROS formation in rainbow trout larvae exposed to low or moderate concentrations of glyphosate was weak or low enough to be detoxified by the antioxidant systems causing no changes in TBARS and protein carbonyls groups.

Among the enzymes involved in ROS detoxification are SOD (superoxide dismutase), CAT (catalase) and GST (glutathione-S-transferase). Inhibition of CAT and SOD activities in liver were observed following exposure to glyphosate by Ferreira (2010) in silver catfish, Modesto and Martinez (2010) in *Prochilodus lineatus* and by Sinhorin (2014) in surubim (*Pseudoplatystoma sp*). In contrast, CAT activity was induced in liver of *L. obtusidens* exposed up to 6 mg/L of Roundup® (a.i.). We observed that *cat* gene was significantly repressed in larvae exposed to 1 mg/L of glyphosate. Topal et al. (2015) studied both gene expression and enzymatic activity in liver of juvenile rainbow trout exposed to different concentrations of glyphosate (from 2.5 to 10 mg/L) from 6 to 96 h, observing that the expressions of *cat* and *sod* were induced the first 6 h and then significantly decreased after 24 h of exposure. In the same study, Topal et al. (2015), observed that the trend of the antioxidant enzymes activity of catalase was opposed to the level of gene expression.

Interestingly, Webster and Santos (2015) studied the transcriptional profile, using RNA-seq, of brown trout females exposed to glyphosate and Roundup (0.01, 0.5 and 10 mg/L) for 14 days. They identified differentially expressed genes that encode antioxidant system proteins (up-regulation of glutathione reductase, *gsr*) stress-responses proteins (heat shock proteins, *ddit*, *ddit4l* and *gadd4l*) and pro-apoptotic signalling proteins (transcription factor tumour suppressor protein *p53*). The nature of the response of the cell depends on the amount and the duration of the stress, since cells respond in a variety of signalling pathways (Fulda et al., 2010; Webster and Santos, 2015). According to Webster and Santos (2015), low concentrations of ROS may help to induce pro-survival signalling, while higher levels of oxidative stress and cellular damage might activate cell death signalling pathways as a protective mechanism. In addition, in this same study (Webster and Santos, 2015), few changes in pro-apoptotic factors were observed suggesting a pro-survival stress response at lower concentrations of glyphosate producing low levels of oxidative stress.

The *cox1* gene code the cytochrome c oxidase subunit 1, which is one of the enzymes involved in the respiratory electron chain transport in mitochondrial membrane. The

602 mitochondrial electron-transport chain is the main source of ROS during normal metabolism
603 (Chen et al., 2003). While cytochrome oxidase is not a source of ROS, its inhibition may
604 promote ROS production (Chen et al., 2003). Our results revealed a significant induction of
605 *cox1* (x2) gene expression on whole larvae exposed to 1 mg/L of glyphosate. An induction of
606 *cox1* could be a cell response to maintain respiratory chain function (Arini et al., 2015).
607 Induction of *cox*1 gene could be viewed as a mechanism by which to restore mitochondria
608 activity and to efficiently consume O_2 and thus to limit ROS production. Induction of *cox1* gene
609 expression could be considered as a mechanism to avoid ROS production (Achard-Joris et al.,
610 2006).
611

612 **Conclusions**

613 This study provides an extensive evaluation of the toxicological effects of glyphosate using an
614 *in vivo* and *in vitro* approach. Results revealed that relatively low concentrations of glyphosate
615 induced hyperactive swimming behavior and morphological cranio-facial alterations onlarvae.
616 In parallel, the studied cell line, RTL-W1, exhibited a DNA damage, which were not observed
617 in blood cells from exposed larvae using the same concentrations of glyphosate. This
618 difference may be explained by the duration of exposure, which was longer, and could have
619 led to an activation of the antioxidant and DNA repair system on blood cells. Decreased TBARS
620 levels and the differential regulation of *cat* and *cox1* gene expression observed on whole
621 exposed larvae could also confirm this hypothesis. It is important to consider the adjuvants in
622 commercial formulations, which can increase the toxicity of glyphosate for vertebrates, and not
623 only the active compound. Regarding the toxicity of glyphosate highlighted in rainbow trout
624 ELS at concentrations that can be found in aquatic ecosystems, we can conclude that
625 glyphosate can pose a potential risk for the most sensitive stage of fish.

626

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FIGURE CAPTIONS

Figure 1. Biometric analyzes of larvae after exposure to 0.1 and 1 mg/L of glyphosate. (A) total body length (mm), (B) head length of larvae (mm) and (C) ratio of head size to total length of larvae (%) are showed. Different letters indicate significant differences between conditions (Mean ± SD N = 3, ANOVA, $p < 0.05$).

Figure 2. Percentage of malformed rainbow trout larvae after 21 days of exposure to glyphosate. Different letters indicate significant differences (Mean ± SD, N = 3, ANOVA, $p<0.05$).

Figure 3. Mean velocity (cm/s) of larvae exposed to glyphosate after a light stimulation. Velocity was recorded after 30 min video tracked analysis. Data was average over each 1 min interval (A) and oer each 10 min (B). Different letters indicate significant differences for each period of illumination (Mean ± SD N = 3, ANOVA, $p < 0.05$).

Figure 4. Cumulative time of high mobility (a) ; mobility (b); and immobility (c) on larvae exposed to glyphosate. Different letters indicate significant differences between each period of time (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

Figure 5. DNA damage in blood cells from rainbow trout larvae after exposure to 0.1 and 1 mg/L of glyphosate, with- and without addition of enzymatic Fpg treatment. Different letters indicate significant differences between treatments (Mean ± SD, N = 3, ANOVA, $p < 0.05$).

Figure 6. Lipid peroxidation (A) expressed as nanomoles of TBARS/mg of protein and protein carbonyls (B) expressed as nanomoles of carbonyl/mg of protein in rainbow trout exposed to glyphosate. Different letters represent significant differences. All values are expressed as Mean ± SD, N=3, ANOVA.

Figure 7. Comparative cytotoxicity of glyphosate (A) and Roundup (B) on the RTL-W1 cell line after 24 h of exposure. Asterisks represent significant differences compared to control. Values represent Mean ± SD. (N=3, Kruskal-Wallis, p<0.05).

Figure 8. DNA damage in RTL-W1 cell line induced by glyphosate (A) and Roundup (B) measured by the comet assay with and without Fpg treatment. Values represent Mean ± SD. Different letters indicate significant differences. N=3, Kruskal-Wallis (p<0.05).

Figure 1.

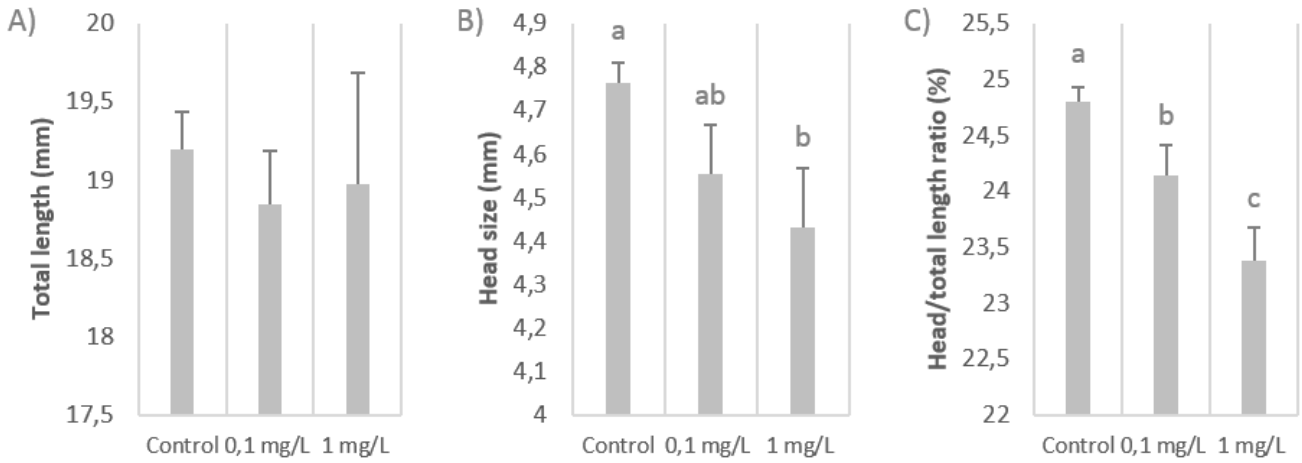
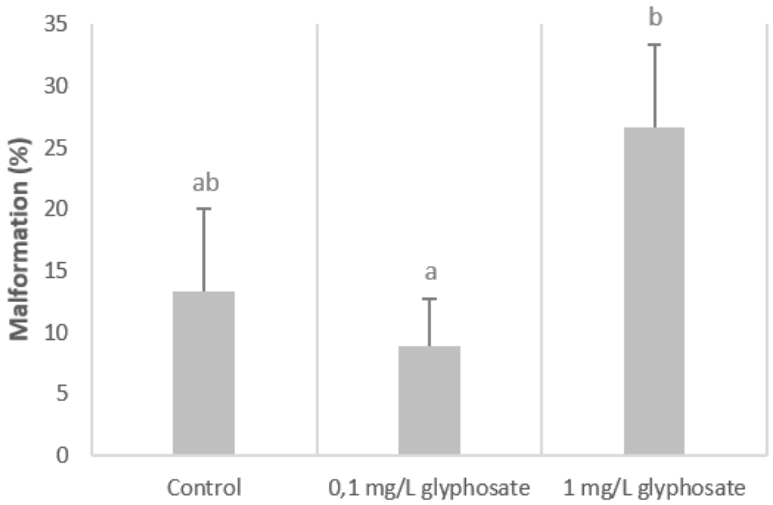
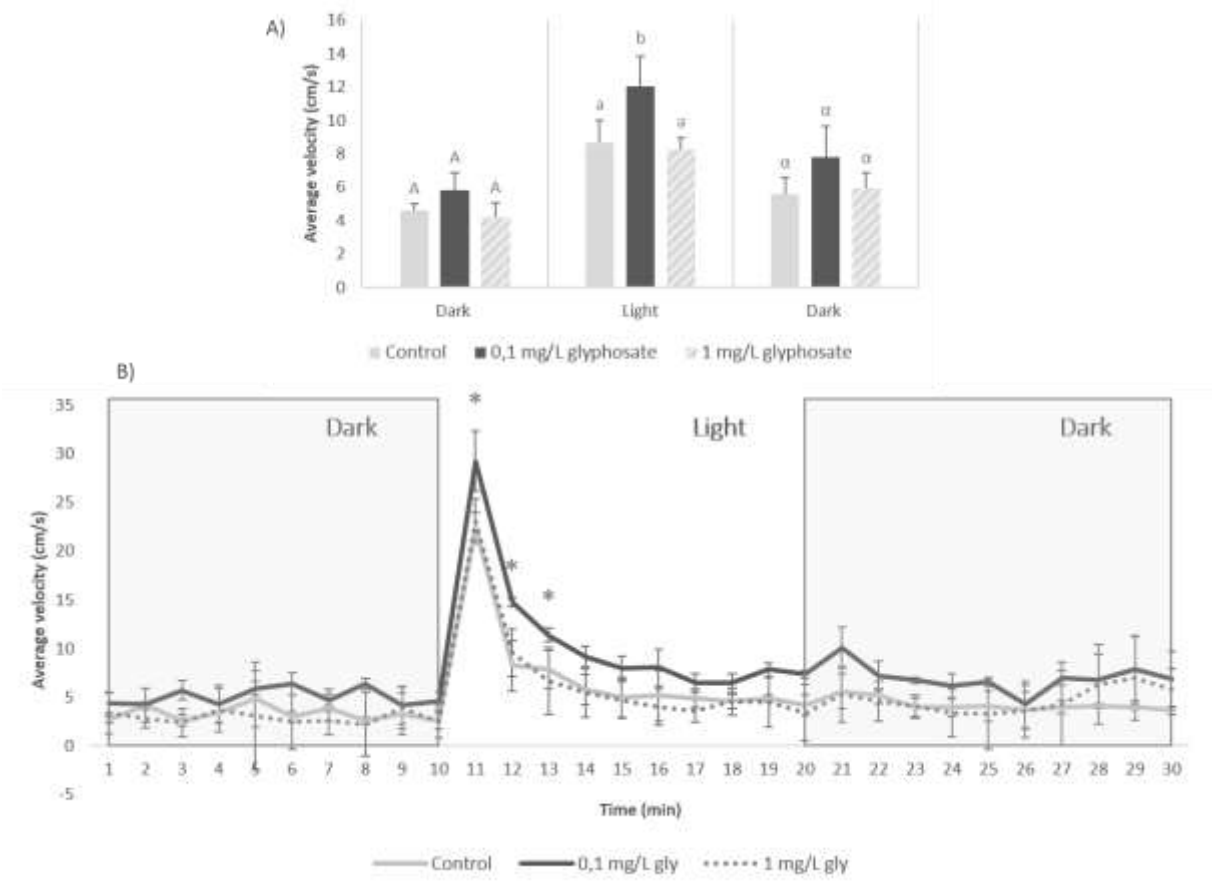


Figure 2.


Malformation (%)
35
30
25
20
15
10
5
0
ab
a
b
Control
0,1 mg/L glyphosate
1 mg/L glyphosate

964 Figure 3.

965



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968 Figure 4.

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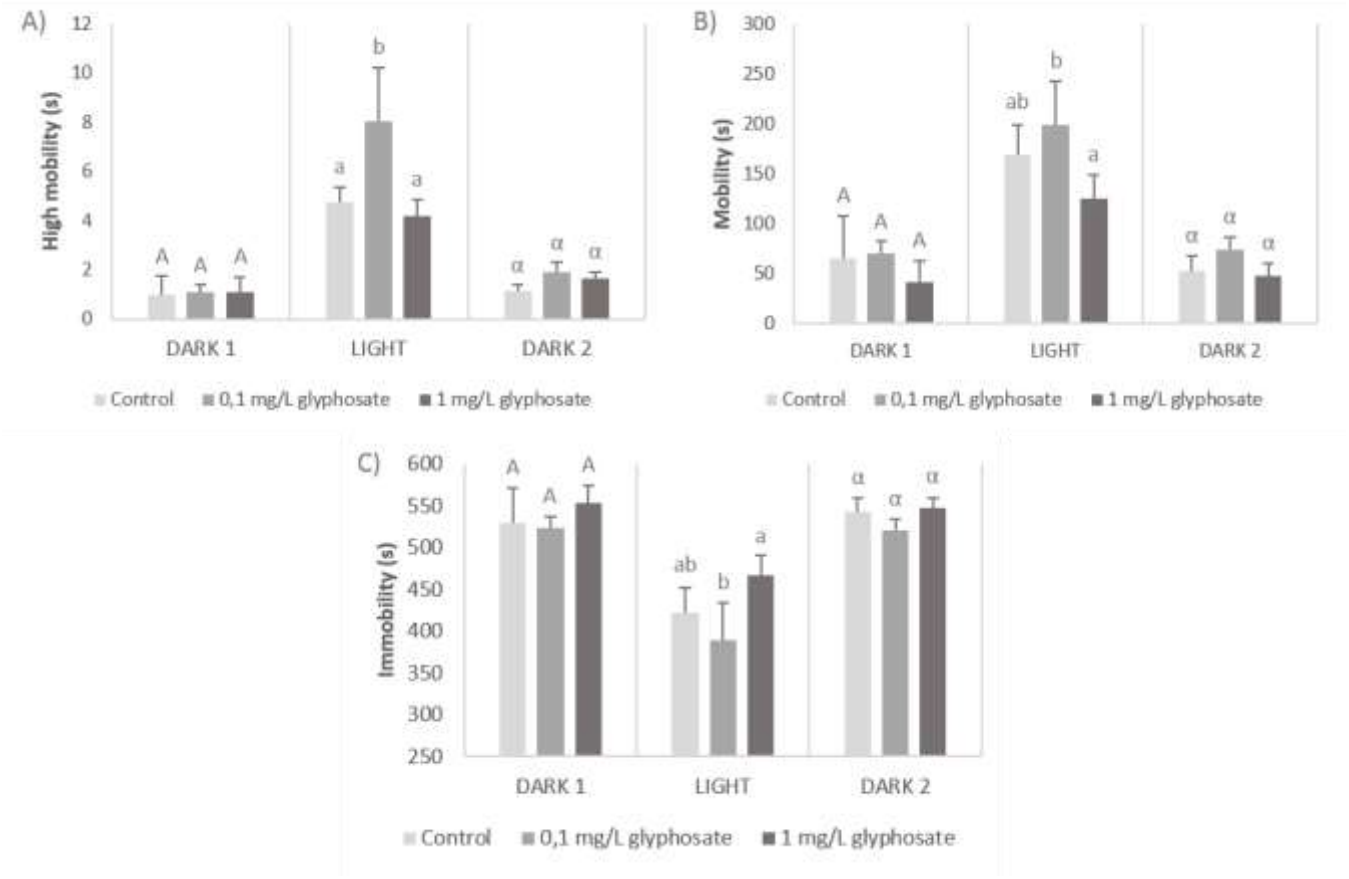

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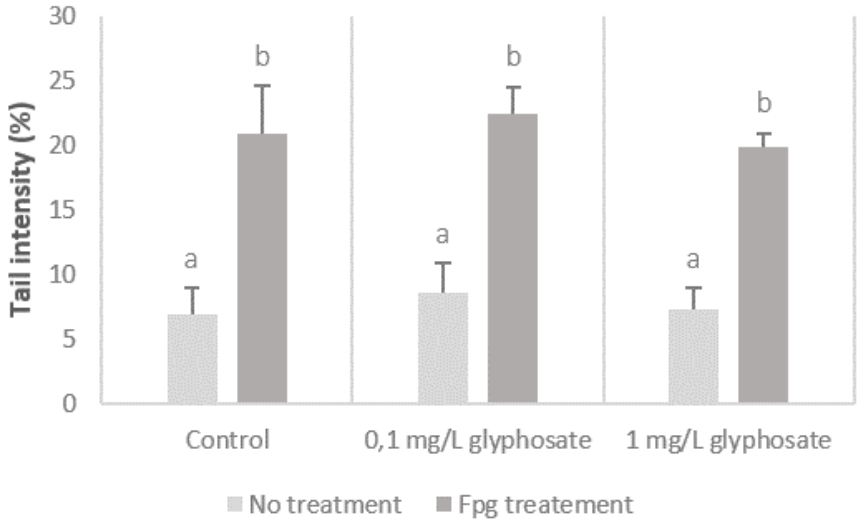
Figure 5.


Tail intensity (%)
30
25
20
15
10
5
0
a
b
a
b
a
b
Control
0,1 mg/L glyphosate
1 mg/L glyphosate
No treatment
Fpg treatement

977 Figure 6.

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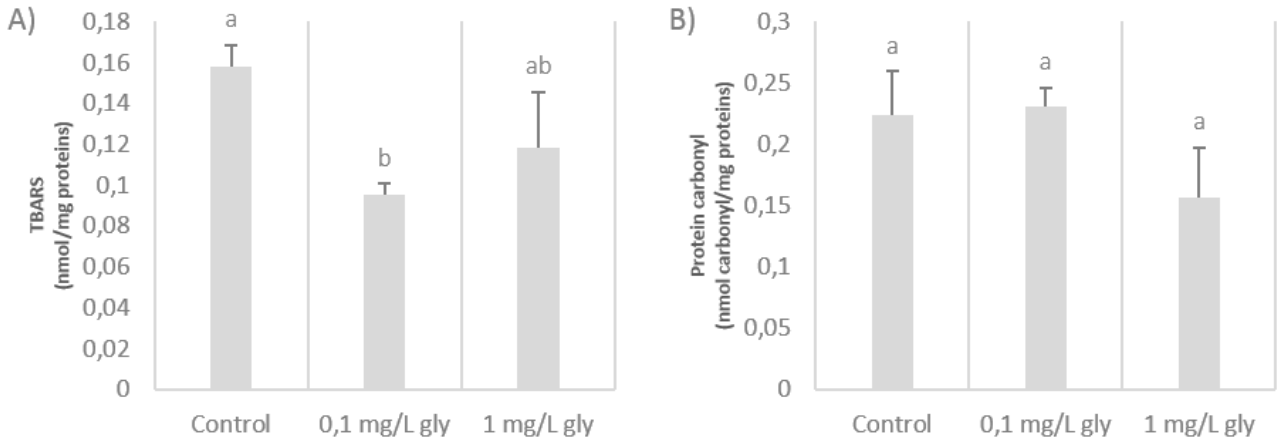

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981 Figure 7.

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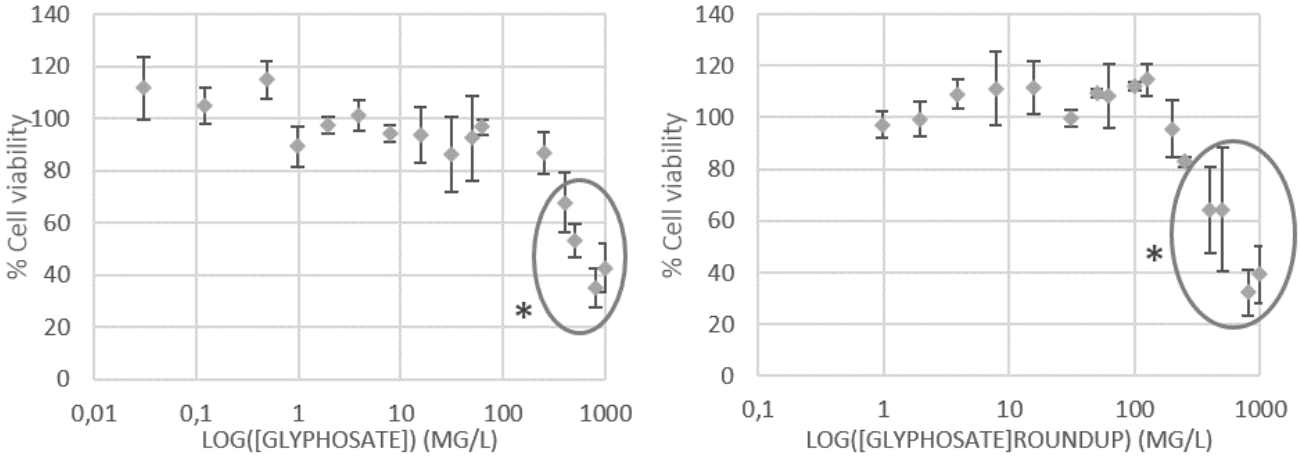

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986 Figure 8.

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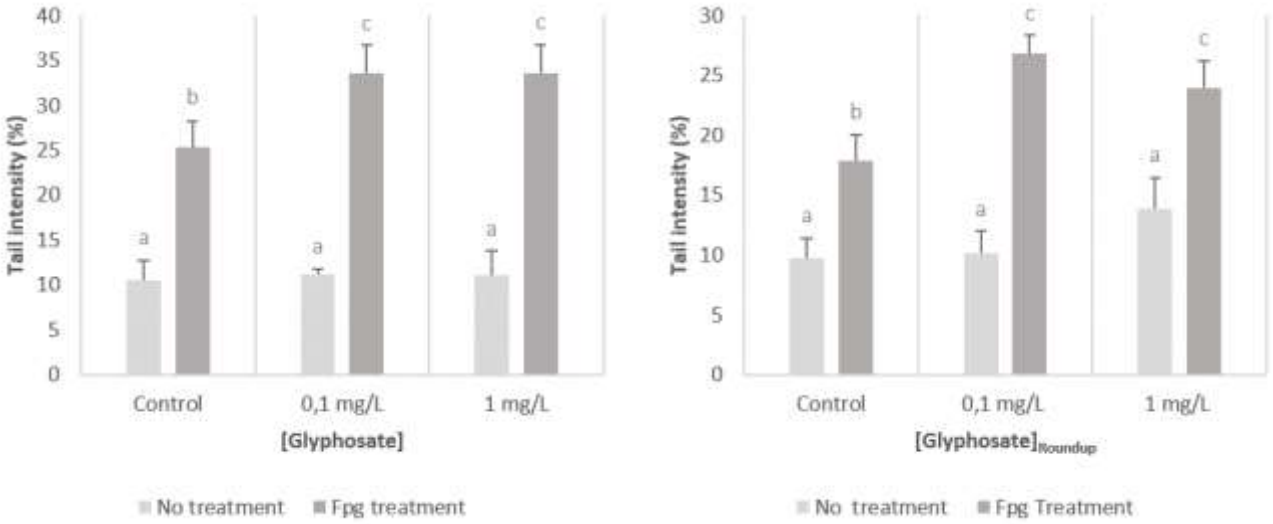

988

989

990

991 **Table 1:** Accession number and specific primer pairs for the Oncorhynchus mykiss used in
992 our study.

Gene	Accession number	Primer (5' – 3')
rpl7	NM_001160672.2	GGTCGCTCTCACAGACAACA[a] TTATGTCCGTCCCTCTGGGT[b]
ef1α	NM_001124339.1	ATGGGCTGGTTCAAGGGATG[a] GATCATACCGGCCTTCAGGG[b]
cat	FJ226382.1	CAGGTGTCTTTCTTGTTCAG[a] GTCCAGGATGGGAAGTTGC[b]
sod	NM_001124329.1	TGATTGGGGAGATCTCGGGT[a] CGGGTCCAGTGAGAGTCAAC[b]
gst	BT073173.1	ATTTTGGGACGGGCTGACA[a] CCTGGTGCTCTGCTCCAGT[b]
er-b	AJ242741	AGCCCTCTCCTCCACCCTACCA[a] ACAGCTGGCTGAGGAGGAGTT[b]
cox1	KP013084.1	TCGTTTGAGCCGTGCTAGTT[a] CTTCTGGGTGGCCGAAGAAT[b]
12s	KY798500.1	GCGCCAGCTTAAAACCCAAA[a] GCCCATTTCTTCCCACCTCA[b]
ogg1	XR_002474791.1	CTGATGGACAAGGCCAGTGT[a] GTAAGGACCCCATGGCTGTC[b]
rad51	XM_021612309.1	AGGCTGGAGGAGGACATCAT[a] GTATTTGAGGGTGGCAGCCT[b]
bax	BT074328.1	CAGAAAACCCAGGGAGGCAT[a] AGAACACATCCTGGGCACAG[b]
cyp19a1	XM_021598638	CTCTCCTCTCATACCTCAGGTT[a] AGAGGAACTGCTGAGTATGAAT[b]

[a]Forward primer

[b]Reverse primer

993

994

995

Table 2: Measured concentration of glyphosate in the exposure water for each studied condition.

Nominal concentration (mg/L)	Measured concentration (mg/L)	
0.0	T0	0.0 ± 0.0
	T48	0.0 ± 0,0
0.1	T0	0.12 ± 0.0
	T48	0.12 ± 0.01
1.0	T0	1.22 ± 0.01
	T48	1.22 ± 0.01

Table 3: Effects on viability and development of rainbow trout during glyphosate exposure. Values represent Mean ± SD (N = 3). The results show no significant difference.

	Control	**Glyphosate 0.1 mg/L**	**Glyphosate 1 mg/L**
Embryonic viability (%)	96.3 ± 2.1	95.3 ± 3.8	95.3 ± 3.5
Larval viability (%)	91.9 ± 3.4	93.2 ± 3.6	92.2 ± 6.2
Cumulative viability (%)	88.6 ± 5.2	88.8 ± 3.6	88.0 ± 8.6
Hatching rate (%)	99.0 ± 0.02	97.6 ± 2.4	95.8 ± 1.8
Development time (DD)	307.9 ± 4.4	311.4 ± 3.1	314.0 ± 6.8