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Developmental effect of parental or direct chronic exposure to environmental concentration of glyphosate on the larvae of rainbow trout, *Oncorhynchus mykiss*

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Abstract

The environmental safety profile of glyphosate, the most commonly used herbicide worldwide, is still a subject of debate and little is known about the generational toxicity of this active substance (AS) and the associated commercial formulations called "glyphosate-based herbicides" (GBHs). This study investigated the impact of parental and direct exposure to 1 µg L^{-1} of glyphosate using the AS alone or one of two GBH formulations (i.e. Roundup Innovert® and Viaglif Jardin®) in the early developmental stages of rainbow trout. Three different modes of exposure on the F1 generation were studied: (1) intergenerational (i.e. fish only exposed through their parents); (2) direct (i.e. fish exposed only directly) and (3) multigenerational (i.e. fish both exposed intergenerationally and directly). The impact of chemical treatments on embryo-larval development (survival, biometry and malformations), swimming behaviour, biochemical markers of oxidative stress equilibrium (TBARS and catalase), acetylcholine esterase (AChE) and energy metabolism (citrate synthase, CS; cytochrome-c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G6PDH) was explored. Chemical exposure did not affect the survival of F1 embryos or malformation rates. Direct exposure to the AS induced some biometric changes, such as reduction in head size (with a 10% decrease in head length), independently of co-formulants. Intergenerational exposure to the AS or the Roundup GBH increased swimming activity of the larvae, with increase of between 78 and 102% in travel speeds. Viaglif co-formulants appear to have counteracted this behavioural change. The minor changes detected in the assayed biochemical markers suggested that observed effects were not due to oxidative damage, AChE inhibition or alterations to energy metabolism. Nonetheless, multi- and intergenerational exposure to Roundup increased CS:CCO and LDH:CS ratios by 46% and 9%, respectively, with a potential modification of the aerobic-to-anaerobic energy production balance. These biochemical effects were not correlated with those observed on individual level of biological organization. Therefore, further studies on generational toxicity of glyphosate and its co-formulants are needed to identify the other mechanisms of glyphosate toxicity at the cellular level.

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Keywords: Glyphosate-based herbicide, Chronic exposure, Embryo-larval development, Generational toxicity, Larval behaviour

1. Introduction

Glyphosate is the most commonly used herbicidal active substance (AS) in the world [6]. Due to its efficiency in controlling weed development, this agrochemical rapidly became vital to the agricultural sector, which has adapted its practices to this herbicide, particularly with the development of genetically modified glyphosate-resistant crops [62, 65]. The constant increase in the use of this herbicide throughout the world over the past years has made it ubiquitous in the environment [11]. Naturally, this widespread use has raised issues on the ecotoxicity of glyphosate, and some studies have evaluated the risk posed by this substance for different environmental compartments [26, 29, 21].

Glyphosate, with a mean half-life from 2.8 to 500.3 days in soils and 6.8 to 21.8 days in the water phase of water-sediment system [21] (the variability of these values is due to the different biotic and abiotic parameters influencing its degradation [29]), does not have a very high level of persistence in the environment [43, 47]. In surface water, its occurrence and concentrations depend on the climate, the agricultural region and the frequencies and the AS doses used [15]. In French surface water, a maximum concentration of $70.2\,\mu g\,L^{-1}$ was detected among the 21.561 sampling points followed in a 10-year period going from 2007 to 2017 the [3]. This was the unique analysis that overwhelmed the reported value of Predicted No Effect Concentration (PNEC of $60\,\mu g\,L^{-1}$, determined using both acute and chronic toxicity values) in 10 years. However lower concentrations were detected in 49.7% of the sampling point in 2017. Also, mean concentration in french surface water reported by Ineris [35] was $0.22\,\mu g\,L^{-1}$ of active substance. Furthermore, several studies have provided evidence that glyphosate associated to co-formulants in glyphosate-based herbicides (GBHs) is toxic to aquatic wildlife [26, 29, 10]. Nevertheless, ecotoxicological research comparing the effect of long-term glyphosate exposure through pure AS or in GBHs in fish are lacking [58].

The bioaccumulation potential of glyphosate in animal tissues is low [18, 21], but some studies have revealed that glyphosate and GBHs can affect physiological processes in fish (e.g. oxidative stress, mitochondrial physiology, immune function, energy metabolism), with severity depending on the life stage and the species considered [31, 52, 37, 67, 68]. Most studies have focused on non-environmentally relevant doses with high toxicity, which trigger unspecific physiological responses, revealing general dysfunctions rather than perturbations directly related to the mode of action of glyphosate. Nonetheless, several ecotoxicological studies have pointed out the toxicity of glyphosate, alone or co-formulated, at lower doses more in line with environmental concentrations [42, 4, 72, 24]. At these environmentally relevant doses, glyphosate alone induces effects at different levels of biological organization

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in fish: e.g. at the cellular level, the inhibition of certain enzymes such as acetylcholine esterase (AChE) [31], changes in parameters related to the oxidant/antioxidant equilibrium [53, 4], modifications in energy metabolism [42, 5, 31] and at the individual level, disruptions in early development [25, 71] and behavioural changes [72, 24, 28, 9].

Toxicity can potentially be transmitted vertically through generations, thereby modulating the toxicity of glyphosate and its co-formulants in the natural environment [33]. The generational toxicity of glyphosate has not been intensively studied, principally due to the complexity of the experimental design which must include several generations of fish. Intergenerational toxicity is defined as the toxicity transmitted from the F0 genitor generation to the F1 generation, with the F1 generation being directly contaminated via the germinal cells produced in the parental bodies [7, 60]. Multigenerational toxicity corresponds to direct exposure, at the level of the entire individual, of both genitors and future generations [33]. In *Danio rerio*, the exposure of the F0 generation to both glyphosate and a GBH at a concentration of $10\,\mathrm{mg\,L^{-1}}$ increased the F1 susceptibility to these pesticides during embryogenesis (i.e. increased mortality rate and premature hatching) [66]. Another study [58], showed that a reduction in hatching success and an increase in developmental abnormalities may be associated with epigenetic effects detected in the parental generation following exposure during their early life stages to glyphosate, co-formulated or not, at the concentration of $0.5\,\mathrm{mg\,L^{-1}}$.

In this study, we evaluated the ability of a low environmental concentration of glyphosate and two GBHs to induce direct and/or generational toxic effects in an F1 generation of rainbow trout (*Oncorhynchus mykiss*). Potential toxicity was investigated at the individual level, with analyses of malformation frequencies and behavioural changes, and at the cellular level, focusing on energy metabolism and oxidative stress.

2. Materials and methods

2.1. Ethics statement

Assays on fish were done in strict accordance with European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). Experimental procedures were validated by the animal ethics committee ANSES/ENVA/UPC No. 16 and authorized by the French Ministry of National Education, Higher Education and Research (APAFIS#2019010812403065). A lethal dose of 100 ppm of eugenol into tank water was used to euthanize fish.

2.2. Chemical compounds

We used the AS glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6) and two GBHs, Roundup Innovert® (R) and Viaglif Jardin® (V). The purity of G was 98%, and the glyphosate concentration of Roundup and Viaglif were 360 and $420\,\mathrm{g\,L^{-1}}$, respectively. The two commercial products were bought at Agrilisa, R was formulated for professional use and V was formulated for home gardens. Details on the formulations and concentrations of these two commercial products were not listed and were unknown because they are proprietary and protected by industrial secret. For each product, aqueous concentrated

solutions (4 mg L^{-1}) were prepared and stored under appropriate conditions (darkness, 4°C). Pure glyphosate was diluted first in 10 mL of pure methanol (solvent concentration of concentrated solution was 10 mL L^{-1} so the final dose of methanol exposure was kept under 4 µL L^{-1} as recommend by Hutchinson et al. [34]).

2.3. Fish

Specific pathogen free mature rainbow trout (3 years old; F0 generation) were exposed daily for 8 months to control (C) or to a mean glyphosate concentration of 123 ng L^{-1} using pure glyphosate, Roundup or Viaglif, before producing the F1 generation [40]. Embryonic development was conducted in a shallow bottom tank containing approximately 300 L. All fish were maintained in filtered river water, with a water flow rate to ensure complete renewal once an hour and maintain approriate physico-chemical conditions and oxygen saturation greater than 60%. A photoperiod of 12 h of daylight was maintained throughout the experiments. Fish feed (Le Gouessant), adapted to fish size, was given *ad libitum*.

2.4. Reproduction and embryonic development

Reproduction of fish from F0 generation is described in the article of Le Du-Carrée et al. [40]. Embryonic development of the F1 generation until the eyed stage [8] was conducted in tanks, containing approximately 300 L, continuously renewed with river water (at a flow rate of approximately 300 L h^{-1}). After the eyed stage, rainbow trout embryos were placed in 10 tanks (40 L) in a confined room. Temperature was maintained at 8 °C ± 2 °C throughout embryonic development.

2.5. Chemical exposure

Once the embryos reached the eyed stage, they were exposed to 10 conditions of chemical exposure that are given in Figure 1. The name of each condition is composed of two letters separated by a slash, the first letter represents the chemical exposure of the F0 generation and the second letter represents the chemical exposure of the F1 generation. Fish from non-contaminated parents and not directly exposed to glyphosate or GBHs formed the control condition (i.e. C/C). Fish from non-contaminated parents, but directly contaminated make up the C/G, C/R, and C/V conditions. Fish from contaminated F0 and not directly contaminated compose the "intergenerationally contaminated" conditions G/C, R/C, and V/C. Finally, the fish that were contaminated directly and intergenerationally represent the "multigenerational exposure" conditions G/G, R/R, and V/V. Chemical exposure of the F1 generation was conducted using the same methodology as that used for the F0 (details are available in ref. [40]): every working day (generally 5 days a week), 10 mL of the condition respective concentrated chemical solution was added to the experimental tanks in which water input was stopped for 1 h. After 1 h of contact, water flow was set to 13.5 L h^{-1} for the rest of the day, resulting in the gradual dilution of glyphosate. The theoretical kinetics of glyphosate concentrations was modelled using the Equation 1 and the resulting curve is shown in Figure 3. The integrated mean daily expected concentration was approximately 123 ng L^{-1} (the area integrated is the blue zone under the theoretical dilution curve in Figure 3).

$$C(t) = C_{initial} \times e^{-rate/V_{tank} \times time} \quad (1)$$

2.6. Samples and sampling dates

The experimental design timeline is presented in Figure A.5 in supplementary data. To measure hatching frequencies, egg survival was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes until all eggs were either dead 34 or hatched. Larval survival was determined on the hatched eggs used for evaluating hatching frequencies; these eggs were placed in plastic breeding boxes 35 days after hatching.

For each condition, 25 larvae were sampled at 320 degree-day (DD ; sampling date S1, see Figure A.5) after 14 days of direct exposure and at 328 DD (S2) for intergenerational exposure. They were placed in a 3% glutaraldehyde solution (described by [48]), at 4 °C until biometric and malformation analyses. At 488 DD (S3), 25 larvae were sampled in each tested condition to measure oxidative stress and metabolic markers. Whole larvae were flash-frozen in liquid nitrogen and stored at −80 °C for future analyses. Proteins were extracted by homogenizing whole larvae in phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor using the tissue homogenizer Precellys 24 (Bertin Technologies, France).

2.7. Determination of glyphosate concentrations in exposure tank

Glyphosate was quantified in water after two months of chemical contamination using a direct competitive ELISA assay (Novakits, ref. 1500086) at two time points in the daily experimental procedure (i.e. just before restarting the water flow (wfr) and 2 hours after wfr), as described in ref. [40]. A total of 250 µL of filtered water was used to conduct the ELISA assay and final absorbance was read at 450 nm on a TECAN's Spark 10M microplate spectrophotometer. The four-parameter log-logistic function, LL.4, of the "drc" R package [57], was used to generate the standard curve. The OD value obtained for the sample was plotted on the quantification standard curve to determine the glyphosate concentration in each sample.

2.8. Biometric index measurements and malformation analyses

Biometric index measurements and malformations were analysed on images of individual larvae taken on a stereo microscope (Leica MZ75) combined with a ToupCam camera (U3CMOS05100KPA). Body and head length, eye diameter and yolk-sac surface were measured using the software ToupView 3.7 (Figure 2a). Malformation frequencies were determined on larvae considering jaw malformations (Figure 2b), yolk-sac oedema (not shown) and spinal curvatures (Figure 2c) as described by Sulukan et al. [61].

2.9. Swimming behaviour analysis

The protocol of photomotor assay was adapted from the study of [68] and applied to swimming larvae (i.e. 855 DD; S4) maintained at 11 °C throughout the experiment. To avoid perturbation due to the circadian rhythm, analyses were done during a maximum duration of 4 h per day for three days. The DanioVision (Noldus, version 12.1) system

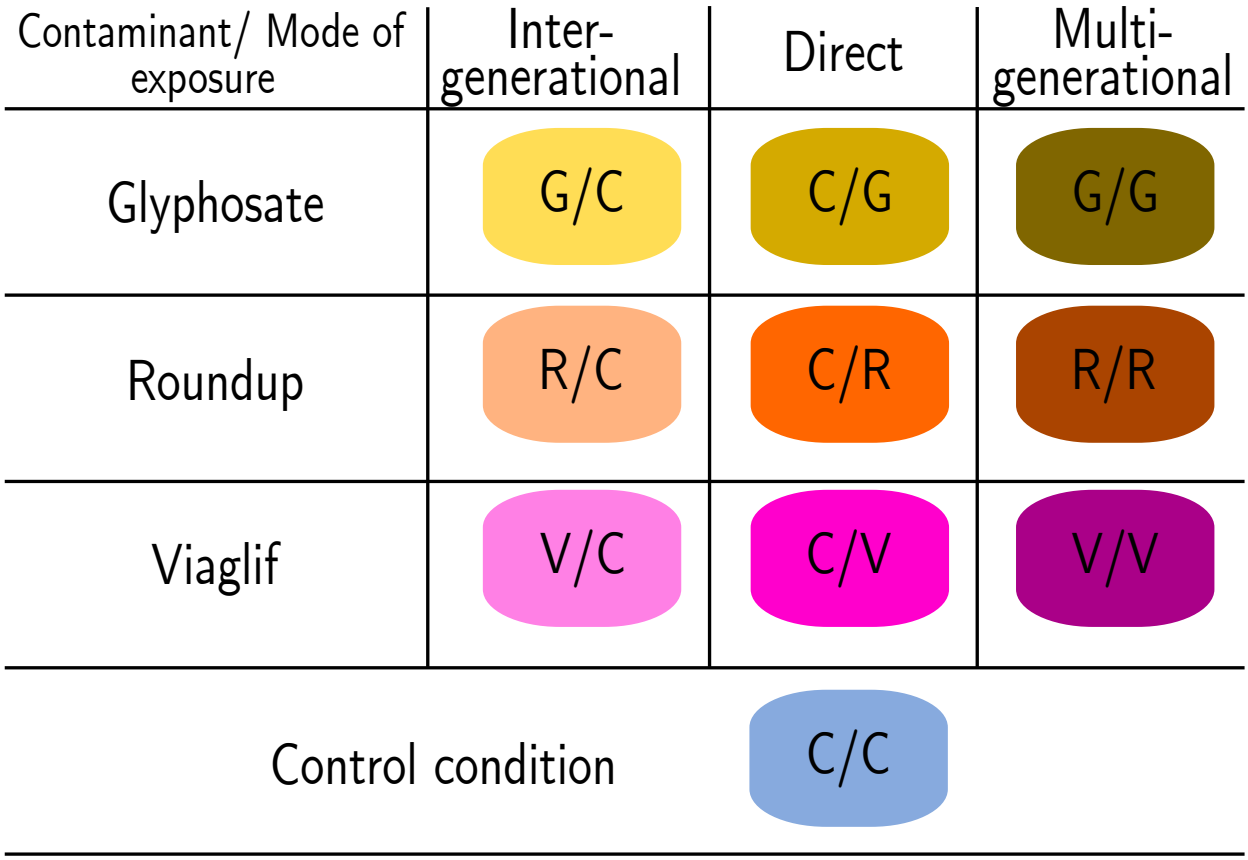
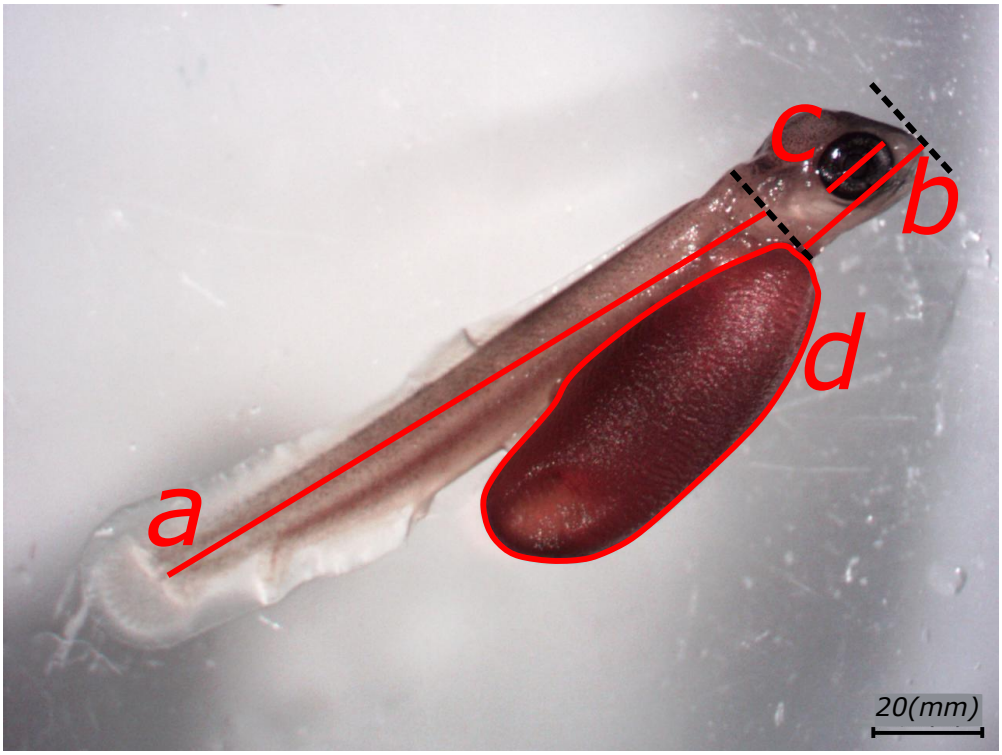
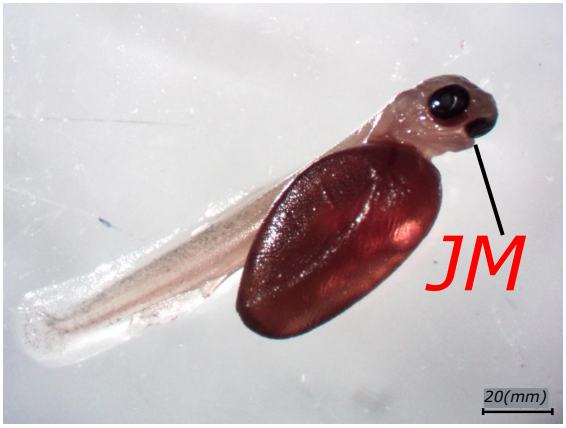


Figure 1: Experimental conditions of chemical exposure for the F1 generation. Parental exposure/offspring exposure; C/C= control/control, G/C= glyphosate/control, C/G= control/glyphosate, G/G= glyphosate/glyphosate, R/C= Roundup/control, C/R= control/glyphosate, R/R=Roundup/Roundup, V/C= Viaglif/control, C/V= control/Viaglif, V/V= Viaglif/Viaglif



(a)



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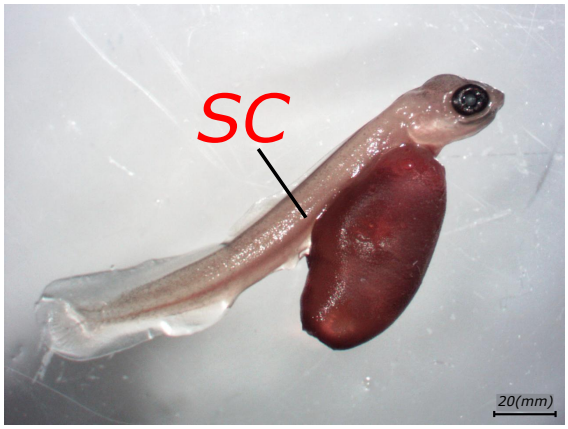

(c)

Figure 2: Normal and malformed larvae. Control larvae (2a) with the biometric indices measured: a. body length; b. head length; c. eye diameter; d. yolk-sac surface. Directly exposed larvae with jaw malformation (JM, from Roundup condition) (2b) and spinal curvature (SC, from Viaglif condition) (2c).

was used to record the behaviour of larvae placed in six-well cell culture plates (Nunc, ref. 140685) with each flat well containing 2 mL of water. For each assay, larvae were acclimated in the plate for 10 min in the dark before the measurements began. An infrared camera coupled to the DanioVision system was used for recording videos. The 30 min recording was divided into three phases at different light intensities: 10 min of darkness (Dark 1), followed by 10 min of light (light 1), finally followed by 10 minutes of darkness (Dark 2). The distance (in meters) travelled by each larva during these three light-darks phases was then used to compare the effect of the different chemical treatments.

2.10. Assays for oxidative stress and metabolic markers and choline esterases

Colorimetric analysis was carried out on a TECAN Spark 10M microplate spectrophotometer. Choline esterases (ChE) were measured at 412 nm, using a protocol adapted from Ellman et al. [22]. Each assay was performed in duplicate or triplicate. Oxidative stress markers, namely thiobarbituric acid reactive substances (TBARS) and catalase (CAT) were assayed in whole larvae homogenates at 532 nm and 240 nm, respectively, following procedures adapted from Espin et al. [23] and Aebi [1]. Metabolic markers, i.e. citrate synthase, CS; cytochrome c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6-phosphate dehydrogenase, G6PDH, were measured in whole larvae samples at 412 nm, 550 nm, 340 nm and 340 nm, respectively, following procedures described in Gauthier et al. [27].

The slope of the optical $density = f(time)$ curve plotted on a calibration curve allowed the calculation of enzymatic activity. Calibration curves were generated using pure enzymes purchased at Sigma-Aldrich: CAT (ref. SRE1010), GPx (ref. G6137), SOD (ref. 55395), AChE (ref. C3389), CA (ref. C2624), CS (ref. C3260), CCO (in-house reference solution made from trout liver at our laboratory, from which enzyme activity was calculated with a molar extinction coefficient, ϵ, of 21.84), LDH (ref. 427217) et G6PDH (ref. G5885) and 1,1,3,3-tetramethoxypropane (ref. T9889) for the TBARS assay. Protein concentrations were measured using the Pierce BCA protein assay kit (ThermoFisher Scientific), and results were expressed as specific activity (IU mg^{-1} of protein).

2.11. Data processing and statistical analyses

Statistical analyses and data processing were done using R software [55]. Figures were generated using the ggplot2 package [70]. Data sets were tested for normality (Shapiro-Wilk) and homoscedasticity (test of Levene for parametric data and Fligner-Killeen for non-parametric data). When normal and homoscedastic data were confirmed, one-way ANOVA tests were used to compare means, followed by Dunnett's post-hoc test [20].For normal and heteroscedastic data, modified one-way ANOVA were employed to compare means [69], followed by Tamhane-Dunnett post-hoc test of [50]. For non-normal data, a Kruskal-Wallis test was used to compare means, followed by Dunn's post-hoc test [19]. Differences between hatching and malformation rates were compared using a chi-squared test. Survival rates for the different chemical treatments were compared using the log-rank test in the "survival" R package [63]. A p-value of 0.05 was used as the threshold for statistical significance.

3. Results

3.1. Glyphosate concentration in water

Glyphosate was never detected in the control tank during the experiment. Glyphosate concentrations comprised between 1.18 ± 0.036 and $1.95 \pm 0.086\,\mu g\,L^{-1}$ were detected in all contaminated tanks 1 h after adding the chemical solutions (Figure 3). Two hours after restarting the water flow, measured concentrations were slightly below those predicted by the theoretical kinetics (from 0.30 ± 0.015 to 0.34 ± 0.021 instead of expected value of$0.51\,\mu g\,L^{-1}$).

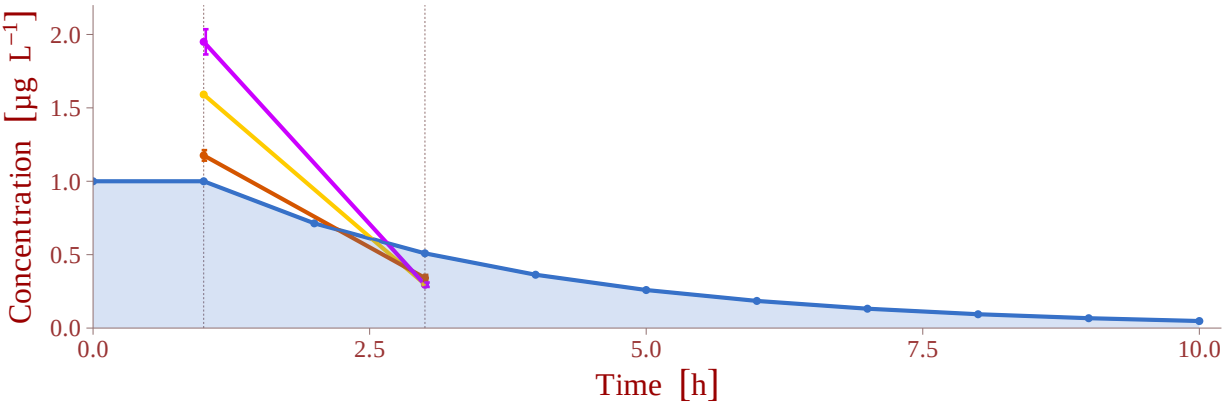

Figure 3: Mean glyphosate concentrations as a function of time ($\mu g\,L^{-1}$). Observed concentrations obtained with the ELISA method were compared with expected concentrations (blue) at different exposure times. The water was sampled after approximately two months after the beginning of the experiment for each condition (glyphosate, yellow; Roundup, orange; Viaglif, purple) just before and 2 h after restarting the water flow. Error bars are standard deviations ($n = 2$).

3.2. Hatching and larvae survival, malformations and biometric indices

Neither the hatching percentage nor the larval survival rate during the 35 days after hatching was significantly affected by chemical exposure (Table 1).

No yolk-sac oedemas were observed. Jaw appeared in larvae at frequencies varying from 0 to 4%, depending on the exposure condition. Spinal curvature was the most frequent malformation observed (12 to 24%). Chemical treatments had no impact on malformation rates (Table 2).

No statistical significant differences between control and directly contaminated larvae were observed for body length, yolk-sac surface or eye:head length ratio (Table 3). However, there were significant reductions in head ($p.value < 0.0001$, d.f.$= 3$ and $f = 9.46$), eye diameter ($p.value < 0.0001$, d.f.$= 3$ and $\chi^2 = 28.06$) and head:body length ratio ($p.value = 0.008$, d.f.$= 3$ and $f = 6.51$). For head length, post-hoc tests revealed significant differences for the C/G (-11%), C/R (-11%) and C/V (-8%) conditions compared with the control ($p.value < 0.05$). For eye diameter, significant differences were observed for the C/G (-11%), C/R (-10%) and C/V (-7%) conditions ($p.value < 0.05$). For head:body length

ratio, significant differences were observed for the C/G (-7%), C/R (-6%) and C/V (-6%) conditions compared with the control ($p.value < 0.05$). Intergenerational exposure induced no statistically significant differences between control and chemically contaminated larvae for most of the biometric indices considered (Table 3). However, a significant effect of chemical concentration was observed in body length ($p.value < 0.0001$, d.f.= 3 and $\chi^2 = 45.05$) and in the ratio between eye and head length ($p.value = 0.0004$, d.f.= 3 and $f = 6.56$). Post-hoc tests revealed significant increases compared with the control, with +7% in body length for the R/C condition and +6% in eye:head length ratio for the V/C condition ($p.value < 0.05$).

3.3. Metabolic activity in whole larvae

Results from the enzymatic and TBARS assays are given in Table 4 (except for CS, CCO and LDH activities presented in supplementary data in Table B.5). No major significant change in mean activity was observed at 488 DD for the oxidative stress markers (i.e. CAT and TBARS), or for AChE and the metabolic markers (i.e. CS, CCO, G6PDH) except for LDH ($p.value = 0.004$, d.f.= 9 and $\chi^2 = 24.02$). A post-hoc test revealed a significant increase in LDH activity for the R/R condition compared with the control (+16%, $p < 0.05$).

Means of CS:CCO ratio values were $8.8 \times 10^{-}3 \pm 5.2 \times 10^{-}4$ for the control condition (Table 4) and comprised between $8.0 \times 10^{-}3 \pm 4.5 \times 10^{-}4$ and $9.7 \times 10^{-}3 \pm 4.2 \times 10^{-}4$ for the other conditions except for the R/R condition which showed a ratio of $13 \times 10^{-}3 \pm 1.7 \times 10^{-}3$. This latter ratio was significantly different to the control condition (global mean difference : $p.value = 0.004$, d.f.= 9 and $\chi^2 = 24.27$; +46%, $p < 0.05$).

For the LDH:CS ratio (Table 4), values were 458.98 ± 7.18 for the control condition, comprised between 458.56 ± 8.42 and 482.19 ± 13.62 for the other conditions, except for R/C and R/R, which showed values of 512.21 ± 10.57 and 501.93 ± 9.09, respectively. For the LDH:CS ratio, a statistical difference was observed among all the conditions ($p.value = 0.002$, d.f.= 9 and $\chi^2 = 25.86$) and a post-hoc test revealed significant differences to the control for the R/C (+12%, $p < 0.05$) and R/R (+9%, $p < 0.05$) conditions.

Table 1: Final hatching rate (expressed in %; $159 \leq n \leq 274$) and survival rate after 35 days (expressed in %; $155 \leq n \leq 268$) for each chemical exposure condition (see Figure 1).

Mode of exposure	Condition	**Parameter**	
		Hatching rate (%)	Survival rate (%)
Control	C/C	95.14	87.92
Direct	C/G	99.11	91.44
	C/R	97.24	93.93
	C/V	97.54	91.70
Intergenerational	G/C	97.04	91.37
	R/C	97.48	90.32
	V/C	96.37	85.48
Multigenerational	G/G	97.81	91.04
	R/R	97.66	91.24
	V/V	94.50	85.19

Table 2: Malformation frequencies measured in trout larvae directly ($23 \leq n \leq 25$) and intergenerationally exposed to contaminants ($n = 25$) for each chemical exposure condition (see Figure 1).

Mode of exposure	Condition	**Type of malformation (%)**	
		jaw	spinal curvature
Direct	C/C	0	13
	C/G	4	12
	C/R	4	12
	C/V	4	24
Intergenerational	C/C	4	12
	G/C	0	12
	R/C	0	12
	V/C	4	20

Table 3: Mean biometric indices measured in trout larvae directly or intergenerationally exposed to contaminants (see Figure 1). Standard errors are given in parentheses under the mean ($23 \leq n \leq 25$). Lengths are expressed in mm, surfaces in mm^2 and ratios in %. The values in bold with an asterisk are significantly different ($p < 0.05$) to the values obtained for the control condition (C/C).

Index	**Intergenerational exposure**				**Direct exposure**			
	C/C	G/C	R/C	V/C	C/C	C/G	C/R	C/V
Body length	12.67 (0.15)	12.52 (0.1)	**13.56*** (0.08)	12.52 (0.14)	13.13 (0.12)	12.61 (0.11)	12.35 (0.16)	12.89 (0.14)
Head length	2.54 (0.046)	2.5 (0.045)	2.67 (0.036)	2.44 (0.046)	2.82 (0.043)	**2.52*** (0.046)	**2.5*** (0.059)	**2.6*** (0.032)
Eye diameter	1.24 (0.025)	1.23 (0.018)	1.3 (0.017)	1.26 (0.02)	1.32 (0.019)	**1.18*** (0.018)	**1.19*** (0.029)	**1.23*** (0.018)
Yolk-sac surface	18.89 (0.59)	17.8 (0.47)	20.21 (0.31)	19.22 (0.72)	19.72 (0.54)	20.14 (0.55)	18.95 (0.72)	20.49 (0.49)
Head:Body length	20.05 (0.22)	19.98 (0.28)	19.7 (0.21)	19.49 (0.29)	21.45 (0.27)	**19.94*** (0.25)	**20.26*** (0.42)	**20.2*** (0.21)
Eye:Head length	48.86 (0.51)	49.45 (0.57)	48.9 (0.44)	**51.64*** (0.52)	47.02 (0.53)	47.00 (0.84)	48.09 (1.21)	47.3 (0.6)

Table 4: Mean specific enzymatic activity, activity ratios and TBARS level measured in whole larvae at 488 DD according to chemical exposure condition (see Figure 1). Standard errors are represented in parentheses under the mean ($12 \leq n \leq 20$). Specific activity is expressed in IU $mg^{-1}$of protein and MDA concentrations in nmol $mg^{-1}$of protein. The numbers in bold with an asterisk are significantly different ($p < 0.05$) to the values observed for the control condition. CCO, cytochrome-c oxidase; CS, citrate synthase; G6PDH, glucose-6-phosphate dehydrogenase; LDH lactate dehydrogenase; CAT, catalase; TBARS, thiobarbituric acid reactive substances, AChE, acetylcholine esterase.

Biochemical marker	**Mode of exposure**									
	Control	**Intergenerational**			**Direct**			**Multigenerational**		
	C/C	G/C	R/C	V/C	C/G	C/R	C/V	G/G	R/R	V/V
CS:CCO	0.0088 (0.00052)	0.0087 (0.00046)	0.0095 (0.00089)	0.0082 (0.00031)	0.0085 (0.00053)	0.0087 (0.00051)	0.0097 (0.00042)	0.008 (0.00045)	**0.013*** (0.0017)	0.0094 (0.00049)
LDH:CS	458.98 (7.18)	471.04 (12.28)	**512.21*** (10.57)	458.56 (8.42)	481.81 (7.65)	480.03 (9.54)	471.29 (10.6)	482.19 (13.62)	**501.93*** (9.09)	470.33 (12.95)
G6PDH	0.64 (0.023)	0.63 (0.033)	0.63 (0.027)	0.59 (0.021)	0.57 (0.018)	0.62 (0.022)	0.65 (0.029)	0.62 (0.038)	0.69 (0.023)	0.61 (0.021)
CAT	56.88 (2.28)	57 (2.49)	58.31 (2.53)	55.26 (2.09)	52.19 (1.99)	56.95 (2.61)	58.15 (3.16)	57.62 (2.8)	62.5 (2.88)	57.61 (1.96)
TBARS	0.35 (0.065)	0.32 (0.027)	0.27 (0.033)	0.24 (0.03)	0.29 (0.036)	0.32 (0.036)	0.35 (0.046)	0.29 (0.024)	0.34 (0.052)	0.44 (0.127)
AChE	0.39 (0.0104)	0.39 (0.011)	0.35 (0.0105)	0.36 (0.0113)	0.37 (0.0106)	0.37 (0.0116)	0.36 (0.0121)	0.42 (0.0244)	0.34 (0.011)	0.36 (0.0085)

3.4. Swimming behaviour of intergenerationally exposed trout

During the photomotor assay, larvae travelled approximately 4.95 m± 0.49 m and 6.86 m± 0.51 m in the first and the second period of darkness, respectively (Figure 4). Speed dramatically decreased in the presence of light with a mean travelled distance of 0.81 m± 0.12 m. No effect of chemical exposure was found in the presence of light, but global mean differences were observed between distance travelled by larvae in the first ($p.value < 0.0001$, d.f.= 3 and $\chi^2 = 30.46$) and in the second period of darkness ($p.value < 0.0001$, d.f.= 3 and $\chi^2 = 41.78$). Post-hoc tests revealed significant differences in means obtained for the G/C and R/C conditions compared with the control during the first period of darkness (+83 and 102%, respectively, $p < 0.05$) and during the second period of darkness (+78 and 83%, respectively, $p < 0.05$).

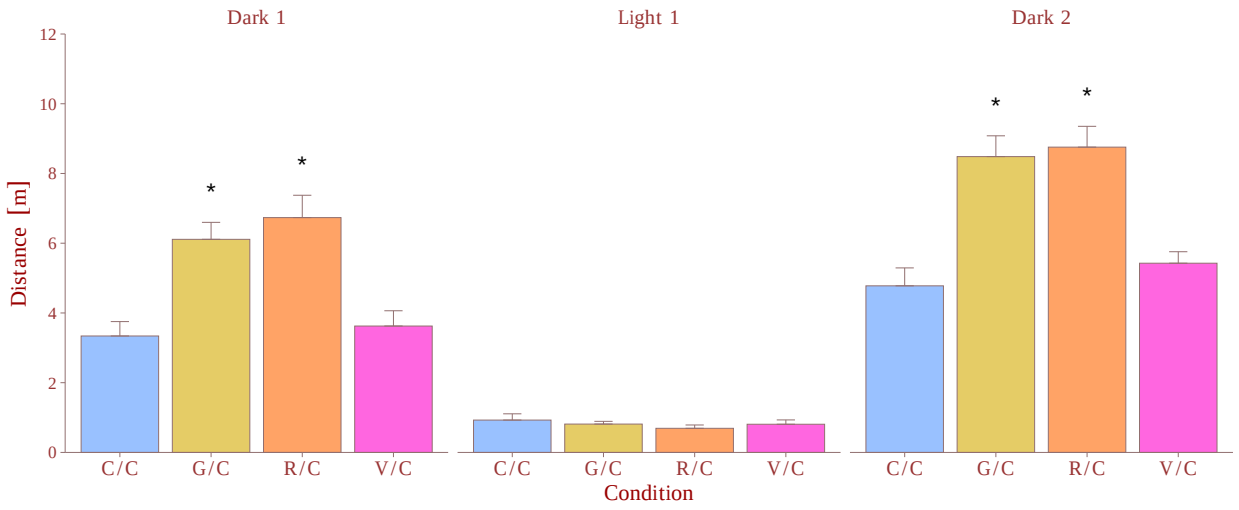

Figure 4: Mean distance (m) travelled by intergenerationnally exposed larvae according to exposure conditions during the different light and dark periods of 10 min. Standard errors are shown at the top of each bar (n= 36). Significant differences to control means are indicated with an asterisk ($p < 0.05$), comparisons were made between groups of a given light or dark period. Parental exposure/direct exposure: C/C= control/control; G/C= glyphosate/control; C/G= control/glyphosate; G/G= glyphosate/glyphosate; R/C= Roundup/control; C/R= control/glyphosate; R/R=Roundup/Roundup; V/C= Viaglif/control; C/V= control/Viaglif; V/V= Viaglif/Viaglif

4. Discussion

Glyphosate is a ubiquitous contaminant of surface water that, with its co-formulants included in GBHs, could be a concern for teleost fish species, particularly because contact occurs during their early development [2]. Also little is known about the generational transmission of the toxicity of these contaminants. This study investigated the effects of chronic exposure to an environmentally relevant concentration of glyphosate (administered pure or in two GBHs) on the development, metabolic markers, and behaviour of juvenile rainbow

trout. To mimic chronic exposure, we added a given quantity of glyphosate once a day to the experiment tanks. At the end of the static exposure period (1 h), the dynamics of glyphosate concentrations in our experimental system showed values higher than nominal concentrations (i.e. where a maximum concentration of $1\,\mu g\,L^{-1}$ is expected). However, two hours after restarting water flow, the observed values were much closer to expected values. Water flow probably better homogenizes the glyphosate in the tank, with dilution over time corresponding to the expected concentration kinetics.

Effects of exposures to glyphosate or GBHs during the embryo-larval development depends on various biotic and abiotic factors such the species considered or doses of exposure. In *Cyprinus carpio* embryos, direct exposure to $5\,\mu g\,L^{-1}$ of glyphosate induced mortality 120 h post-fertilization (hpf) Fiorino et al. [25], whereas in *D. rerio* embryos, only a dose of $50\,\mu g\,L^{-1}$ causes mortality 48 hpf. In another study [66], only multigenerational contamination of $10\,mg\,L^{-1}$ of glyphosate and a GBH reduced embryo survival of *D. rerio*, with no effect for intergenerational exposure at the same concentration or both modes of exposures at lower concentrations (down to $10\,\mu g\,L^{-1}$). Also, direct exposure to glyphosate or GBH did not induce mortality of *D. rerio* or *O. mykiss* during embryo-larval under $1\,mg\,L^{-1}$ of active substance [9, 68]. In the present study, none of the chemical contaminants nor the mode of exposure studied affected the hatching percentages or larval survival. The concentration used, although environmentally relevant, was probably too low to induce mortality in *O. mykiss* embryos or larvae. Also, sublethal concentrations of glyphosate could induce malformations or modification of biometric parameters of fish larvae. In *D. rerio*, doses of up to $50\,\mu g\,L^{-1}$ did not induce malformations in embryos, but there were modifications of biometric body parameters at doses higher than $100\,mg\,L^{-1}$ [72]. At these concentrations, decreases in body length, head and eye area have been detected. Although doses ranging from 0.1 to $10\,mg\,L^{-1}$ do not induce changes at the individual organism level, modifications in the expression of genes involved in embryonic development have been detected at $10\,mg\,L^{-1}$ [72]. Our results did not indicate that glyphosate alone or associated with co-formulants can induce malformations at a concentration of $1\,\mu g\,L^{-1}$. However, some changes in biometric indices were observed depending on the mode of exposure considered. Larvae exposed directly to glyphosate or either of the two GBHs showed a decrease in head size relative to the rest of the body as well as a reduction in eye diameter. These results corroborate those obtained [72] on *D. rerio* with concentrations greater than $100\,mg\,L^{-1}$, suggesting that *O. mykiss* is more sensitive than *D. rerio*. Furthermore, our results reflect those on rainbow trout exposed at an early stage to a GBH at concentrations of 0.1 and $1\,mg\,L^{-1}$ Weeks Santos et al. [68], with no change in total size but a decrease in head size. In our study, direct exposure to the two GBHs and thus their co-formulants did not modulate or increase glyphosate effects on rainbow trout development. Also, whereas intergenerational exposure did not induce as many changes as direct exposure, small developmental modifications were observed, e.g. increased body size and a modified eye to head ratio depending on the GBH. Therefore, exposure of the F1 generation to GBHs at the germinal cell stage or inherited non-genetic changes from contaminated parents (e.g. modified DNA methylation) may have engendered the 2 observed effects [60]. Higher AS concentrations ($0.5\,mg\,L^{-1}$ pure AS) can induce developmental malformations in *Oryzias latipes* upon intergenerational

chronic exposure of an F0 generation, whereas 0.5 and 5 mg L^{-1} of the AS co-formulated in a GBH does not induce any effect [58]. This observation indicates that, depending on the experimental conditions and the species considered, glyphosate can affect development through parental exposure and that co-formulants may modulate this effect.

We investigated the intergenerational impact of glyphosate on swimming behaviour. Direct exposure to glyphosate, alone or co-formulated, has been shown to modify several behavioural traits (e.g. related to feeding, predator avoidance, locomotion) in multiple fish species and at different concentrations [72, 24, 68, 64, 14, 28]. Parental exposure to environmental stressors (i.e. intergenerational stress) including pesticide contamination, have been shown to induce behavioural changes in offspring [13, 38]. In this study, glyphosate induced in particular an increase of swimming activity in larvae in the dark. This effect was observed for pure glyphosate and Roundup, but not for Viaglif, indicating that co-formulants may modulate AS toxicity. The possible explanations, e.g. the existence of one or more co-formulants in Viaglif that may have an antagonistic effect on the AS or decrease its bioavailability, could not be tested because the qualitative and quantitative formulation of the commercial product is undisclosed. Effects observed for glyphosate and Roundup are in accordance with a previous study with doses ranging from 0.01 to 1 mg L^{-1} Zhang et al. [72]. Interestingly, concentrations of up to 10 mg L^{-1} were tested and induced fewer effects, indicating a non-monotonic dose-response of glyphosate and its co-formulants. However, another study reported an increase in the mean speed of O. mykiss larvae exposed during early development to a GBH with glyphosate at 1 mg L^{-1}, with no effect at 0.1 mg L^{-1} [68]. In light of that study and our study, these results may indicate that direct and intergenerational exposure induces similar behavioural changes, but exposure of the parental generation has more potential to modify behaviour of the F1 generation than direct exposure.

The appearance of a toxic effect in fish exposed to glyphosate is correlated with changes in several biochemical markers [31, 53, 4, 42, 5, 68]. Our results have shown that, regardless of the mode of exposure or the contaminants, AChE activity was not affected in larvae. Numerous studies have reported inhibition induced by the pure glyphosate or GBHs at doses comprised between 1 and 30 mg L^{-1} [44] and 0.2 and 20 mg L^{-1}, respectively [12, 46, 30, 31]. However, these effects are not systematically observed in larvae. In *Hypomesus transpacificus* adults, no change was observed after exposure to doses ranging from 0.078 to 896 mg L^{-1} Jin et al. [36]. It is therefore possible that, despite different modes of exposure, the concentration used in this study was too low to induce detectable changes in AChE activity.

Although oxidative stress is frequently involved in pesticide toxicity in fish [41], only a few studies have investigated the disruption of the redox balance potentially induced by glyphosate. Certain changes in oxidative stress-related markers in fish exposed to an AS concentration as low as 0.71 mg L^{-1} have been reported [36, 59]. Studies on GBHs have detected reactive oxygen species (ROS) [56], biochemical modifications (e.g. glutathione levels, enzyme activity) [53, 4, 32], and oxidative damage (e.g. lipid peroxidation, protein carbonylation, DNA damage) [4, 32, 45]. Concentrations inducing effects are generally high, although alterations have been observed in fish at concentrations ranging from 26.5 to 116 µg L^{-1} [53, 4, 32]. We did not detect lipid peroxidation or any increase in catalase activity, which can indicate oxidative damage or an antioxidant response in larvae, respectively.

Our results are in accordance with those of Lanzarin et al. [39] obtained on D. rerio exposed to low doses of a GBH (between 2 to 8.5 µg L^{-1} of glyphosate) during embryonic development, with no effect on ROS production, antioxidant enzymes (i.e. superoxide dismutase, CAT, and glutathione S-transferase), glutathione levels or lipid peroxidation. All these results strongly suggest that the concentration we used was too low to activate antioxidant defences or generate oxidative damage in trout larvae.

Other physiological disruptions in fish, such as changes in the energy metabolism, are also commonly observed during exposure to glyphosate or GBHs [2]. In our experiment, we assayed CCO, CS, G6PDH and LDH activity, because they reflect potential changes in the aerobic metabolism (CCO, CS) [49], anabolism (G6PDH) [51] and anaerobic metabolism (LDH) [16]. We detected only an increase in LDH activity in fish exposed multigenerationally to Roundup. The exposure of the F0 and F1 generation to this GBH may have triggered an increase in anaerobic metabolism to cope with the additional energy demand. A similar change was observed in *Clarias gariepinus* chronically and directly exposed to concentrations ranging from 26.5 to 106 µg L^{-1} of a GBH [5]. We also showed an increase in the CS:CCO ratio of larvae exposed multigenerationally to Roundup, reflecting the potential disruption of the mitochondrial membranes, which may be due to the inhibition of CCO or to the degradation of the lipid membranes by contaminants [17]. The significant increase in the LDH:CS ratio in larvae exposed intergenerationally or multigenerationally to Roundup may indicate that parental exposure induced a need to produce more energy via anabolism rather than aerobic metabolism [54].

5. Conclusions

In this study, we studied the effect of parental and/or direct exposure to an environmental concentration of glyphosate, focusing on the embryo-larval development of the F1 generation. Although no effect was shown on embryo or larval survival rates regardless of the mode of exposure or the contaminants considered, some effects were observed at different levels of biological organization. Glyphosate, apparently independently of co-formulants and only in the case of direct exposure, appeared to induce developmental changes, such as reductions in head size and that of associated organs, but did not cause developmental malformations. Intergenerational exposure to glyphosate increased the swimming activity in larvae, without any correlation with AChE inhibition. This behavioural change may potentially have an important impact on larvae survival in a stressful natural environment. Viaglif co-formulants seemed to counteract this behavioural change. No oxidative stress response or damage was detected in rainbow trout larvae. Nonetheless, parental exposure to one of the two tested GBHs may modify energy production by increasing the amount of energy produced via anaerobic metabolism compared with that produced by aerobic metabolism. Also, multigenerational exposure to the same GBH may potentially cause a reduction in the capacity of mitochondria for energy production. These effects, not detected with the AS alone, were probably not responsible for the other effects observed. Thus, there are likely other mechanisms of glyphosate toxicity at the cellular level and further investigations are needed to fully understand how they are responsible for both direct and generational toxicity

of glyphosate and its co-formulants.

Competing interests

The authors declare that they have no conflict of interest.

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Appendix A. Timeline of the study

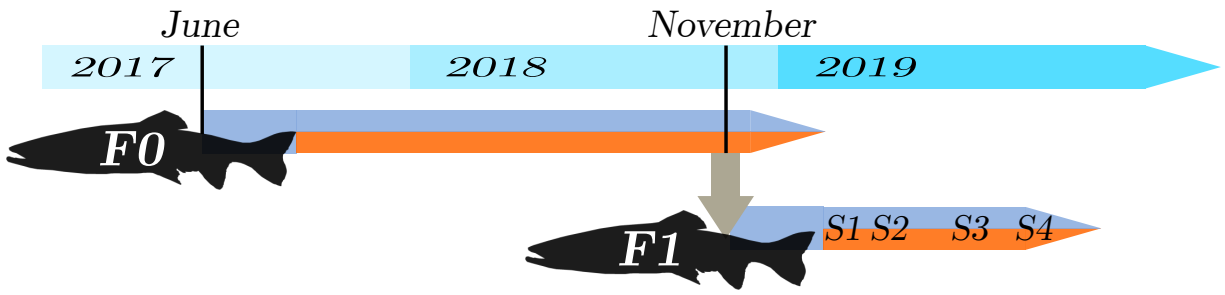

Figure A.5: Timeline of the experiment on generations F0 2014 and F1 2018. Orange and blue lines represent phases with and without chemical exposure, respectively. Dual coloured bars indicate both intergenerational and multigenerational conditions. Biometric analyses on larvae were done at 320 DD (S1) and 328 DD (S2). Swimming larvae were sampled for enzymatic tests at 488 DD (S3). Behavioural analyses began at 855 DD (S4).

Appendix B. Aerobic and anaerobic enzyme activities in whole larvae

Table B.5: Mean specific enzymatic activity measured in whole larvae at 488 DD according to chemical exposure condition (see Figure 1). Standard errors are represented in parentheses under the mean ($12 \leq n \leq 20$). Specific activity is expressed in IU $mg^{-1}$of protein. The numbers in bold with an asterisk are significantly different ($p < 0.05$) to the values observed for the control condition. CCO, cytochrome-c oxidase; CS, citrate synthase; G6PDH, glucose-6-phosphate dehydrogenase; LDH lactate dehydrogenase; CAT, catalase; TBARS, thiobarbituric acid reactive substances, AChE, acetylcholine esterase.

Biochemical marker	**Mode of exposure**									
	Control	**Intergenerational**			**Direct**			**Multigenerational**		
	C/C	G/C	R/C	V/C	C/G	C/R	C/V	G/G	R/R	V/V
CCO	35.77 (1.82)	35.1 (1.74)	32.97 (1.94)	36.04 (1.33)	33.54 (1.91)	34.3 (1.53)	31.21 (1.27)	37.41 (2.24)	29.22 (2.21)	32.79 (1.27)
CS	0.29 (0.008)	0.29 (0.008)	0.29 (0.0073)	0.29 (0.0075)	0.27 (0.0083)	0.28 (0.0058)	0.29 (0.0107)	0.28 (0.0123)	0.32 (0.0121)	0.3 (0.0096)
LDH	134 (4.11)	134.86 (4.58)	146.14 (3.76)	131.3 (3.19)	131.25 (4.2)	136.73 (4.19)	136.62 (4.42)	136.63 (5.47)	**156.08*** (5.21)	139.4 (4.61)

References

[1] Aebi, H., 1984. Catalase in vitro, in: Methods in Enzymology. Elsevier. volume 105, pp. 121–126. doi:10.1016/s0076-6879(84)05016-3.

[2] Annett, R., Habibi, H.R., Hontela, A., 2014. Impact of glyphosate and glyphosate-based herbicides on the freshwater environment. Journal of Applied Toxicology 34, 458–479. doi:10.1002/jat.2997.

[3] Anses, 2019. Synthèse des données de surveillance - Appui scientifique et technique numéro 2017-04. Technical Report. Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Anses). URL: https://www.anses.fr/fr/system/files/Fiche_PPV_Glyphosate.pdf.

[4] Ayanda, I.O., 2018. Toxicity of Sublethal Concentrations of Glyphosate and Paraquat Herbicide in the African Catfish *Clarias gariepinus*. International Journal of Agriculture and Biology 20. doi:10.17957/ijab/15.0642.

[5] Ayanda, O., Oniye, S., Auta, J., Ajibola, V., Bello, O., 2015. Responses of the African catfish *Clarias gariepinus* to long-term exposure to glyphosate- and paraquat-based herbicides. African Journal of Aquatic Science 40, 261–267. doi:10.2989/16085914.2015.1074882.

[6] Benbrook, C.M., 2016. Trends in glyphosate herbicide use in the United States and globally. Environmental Sciences Europe 28, 3. doi:10.1186/s12302-016-0070-0.

[7] Best, C., Ikert, H., Kostyniuk, D.J., Craig, P.M., Navarro-Martin, L., Marandel, L., Mennigen, J.A., 2018. Epigenetics in teleost fish: From molecular mechanisms to physiological phenotypes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 224, 210–244. doi:10.1016/j.cbpb.2018.01.006.

[8] Bobe, J., Andre, S., Fauconneau, B., 2000. Embryonic muscle development in rainbow trout (*Oncorhynchus mykiss*): A scanning electron microscopy and immunohistological study. Journal of Experimental Zoology 286, 379–389. doi:https://doi.org/10.1002/(SICI)1097-010X(20000301)286:4<379::AID-JEZ6>3.0.CO;2-2.

[9] Bridi, D., Altenhofen, S., Gonzalez, J.B., Reolon, G.K., Bonan, C.D., 2017. Glyphosate and Roundup® alter morphology and behavior in zebrafish. Toxicology 392, 32–39. doi:10.1016/j.tox.2017.10.007.

[10] de Brito Rodrigues, L., Costa, G.G., Thá, E.L., da Silva, L.R., de Oliveira, R., Leme, D.M., Cestari, M.M., Grisolia, C.K., Valadares, M.C., de Oliveira, G.A.R., 2019. Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 842, 94–101. doi:10.1016/j.mrgentox.2019.05.002.

[11] Bruggen, A.V., He, M., Shin, K., Mai, V., Jeong, K., Finckh, M., Morris, J., 2018. Environmental and health effects of the herbicide glyphosate. Science of the Total Environment 616-617, 255–268. doi:10.1016/j.scitotenv.2017.10.309.

[12] Cattaneo, R., Clasen, B., Loro, V.L., de Menezes, C.C., Pretto, A., Baldisserotto, B., Santi, A., de Avila, L.A., 2011. Toxicological Responses of *Cyprinus carpio* Exposed to a Commercial Formulation Containing Glyphosate. Bulletin of Environment Contamination and Toxicology 87, 597–602. doi:10.1007/s00128-011-0396-7.

[13] Colson, V., Cousture, M., Damasceno, D., Valotaire, C., Nguyen, T., Cam, A.L., Bobe, J., 2019. Maternal temperature exposure impairs emotional and cognitive responses and triggers dysregulation of neurodevelopment genes in fish. PeerJ 7, e6338. doi:10.7717/peerj.6338.

[14] da Costa Chaulet, F., de Alcantara Barcellos, H.H., Fior, D., Pompermaier, A., Koakoski, G., da Rosa, J.G.S., Fagundes, M., Barcellos, L.J.G., 2019. Glyphosate- and Fipronil-Based Agrochemicals and Their Mixtures Change Zebrafish Behavior. Archives of Environmental Contamination and Toxicology 77, 443–451. doi:10.1007/s00244-019-00644-7.

[15] Coupe, R.H., Kalkhoff, S.J., Capel, P.D., Gregoire, C., 2011. Fate and transport of glyphosate and aminomethylphosphonic acid in surface waters of agricultural basins. Pest Management Science 68, 16–30. doi:10.1002/ps.2212.

[16] Couture, P., Kumar, P.R., 2003. Impairment of metabolic capacities in copper and cadmium contaminated wild yellow perch (*Perca flavescens*). Aquatic Toxicology 64, 107–120. doi:10.1016/s0166-445x(03)00028-6.

[17] Craig, P.M., Wood, C.M., McClelland, G.B., 2007. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 293, R1882–R1892. doi:10.1152/ajpregu.00383.2007.

[18] Duke, S.O., 2020. Glyphosate: environmental fate and impact. Weed Science 68, 201–207. doi:10.1017/wsc.2019.28.

[19] Dunn, O.J., 1964. Multiple Comparisons Using Rank Sums. Technometrics 6, 241–252. doi:10.1080/00401706.1964.10490181.

[20] Dunnett, C.W., 1955. A Multiple Comparison Procedure for Comparing Several Treatments with a Control. Journal of the American Statistical Association 50, 1096–1121. doi:10.1080/01621459.1955.10501294.

[21] (EFSA), E.F.S.A., 2015. Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. EFSA Journal 13, 4302. doi:10.2903/j.efsa.2015.4302.

[22] Ellman, G.L., Courtney, K., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88–95. doi:10.1016/0006-2952(61)90145-9.

[23] Espin, S., Sanchez Virosta, P., García-Fernández, A., Eeva, T., 2017. A microplate adaptation of the thiobarbituric acid reactive substances assay to determine lipid peroxidation fluorometrically in small sample volumes. Revista de Toxicologia 34, 94–98. URL: https://rev.aetox.es/wp/index.php/a-microplate-adaptation/.

[24] Faria, M., Wu, X., Luja-Mondragón, M., Prats, E., Gómez-Oliván, L.M., Piña, B., Raldúa, D., 2020. Screening anti-predator behaviour in fish larvae exposed to environmental pollutants. Science of the Total Environment 714, 136759. doi:10.1016/j.scitotenv.2020.136759.

[25] Fiorino, E., Sehonova, P., Plhalova, L., Blahova, J., Svobodova, Z., Faggio, C., 2018. Effects of glyphosate on early life stages: comparison between *Cyprinus carpio* and *Danio rerio* . Environmental Science and Pollution Research 25, 8542–8549. doi:10.1007/s11356-017-1141-5.

[26] Folmar, L.C., Sanders, H.O., Julin, A.M., 1979. Toxicity of the herbicide glyphosate and several of its formulations to fish and aquatic invertebrates. Archives of Environmental Contamination and Toxicology 8, 269–278. doi:10.1007/BF01056243.

[27] Gauthier, C., Campbell, P.G., Couture, P., 2008. Physiological correlates of growth and condition in the yellow perch (*Perca flavescens*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 151, 526–532. doi:10.1016/j.cbpa.2008.07.010.

[28] Giaquinto, P.C., de Sá, M.B., Sugihara, V.S., Gonçalves, B.B., Delício, H.C., Barki, A., 2017. Effects of Glyphosate-Based Herbicide Sub-Lethal Concentrations on Fish Feeding Behavior. Bulletin of Environment Contamination and Toxicology 98, 460–464. doi:10.1007/s00128-017-2037-2.

[29] Giesy, J.P., Dobson, S., Solomon, K.R., 2000. Ecotoxicological Risk Assessment for Roundup® Herbicide, in: Reviews of Environmental Contamination and Toxicology. Springer New York. volume 167, pp. 35–120. doi:10.1007/978-1-4612-1156-3_2.

[30] Glusczak, L., dos Santos Miron, D., Crestani, M., da Fonseca, M.B., de Araújo Pedron, F., Duarte, M.F., Vieira, V.L.P., 2006. Effect of glyphosate herbicide on acetylcholinesterase activity and metabolic and hematological parameters in piava (*Leporinus obtusidens*). Ecotoxicology and Environmental Safety 65, 237–241. doi:10.1016/j.ecoenv.2005.07.017.

[31] Glusczak, L., dos Santos Miron, D., Moraes, B.S., Simões, R.R., Schetinger, M.R.C., Morsch, V.M., Loro, V.L., 2007. Acute effects of glyphosate herbicide on metabolic and enzymatic parameters of silver catfish (*Rhamdia quelen*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 146, 519–524. doi:10.1016/j.cbpc.2007.06.004.

[32] Guilherme, S., Gaivão, I., Santos, M., Pacheco, M., 2012. DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide - Elucidation of organ-specificity and the role of oxidative stress. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 743, 1–9. doi:10.1016/j.mrgentox.2011.10.017.

[33] Hanson, M.A., Skinner, M.K., 2016. Developmental origins of epigenetic transgenerational inheritance. Environmental Epigenetics 2, dvw002. doi:10.1093/eep/dvw002.

[34] Hutchinson, T., Shillabeer, N., Winter, M., Pickford, D., 2006. Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. Aquatic Toxicology 76, 69–92. URL: https://www.sciencedirect.com/science/article/pii/S0166445X05003255, doi:https://doi.org/10.1016/j.aquatox.2005.09.008.

[35] Ineris, 2020. GLYPHOSATE ET SES PRINCIPAUX COMPOSES. Technical Report. Verneuil-en-Halatte : Institut national de l'environnement industriel et des risques (Ineris) - 181229 - v2.0, 27/03/2020. URL: https://www.inrs.fr/dms/ficheTox/FicheFicheTox/FICHETOX_273-1/FicheTox_273.pdf.

[36] Jin, J., Kurobe, T., Ramírez-Duarte, W.F., Bolotaolo, M.B., Lam, C.H., Pandey, P.K., Hung, T.C., Stillway, M.E., Zweig, L., Caudill, J., Lin, L., Teh, S.J., 2018. Sub-lethal effects of herbicides penoxsulam, imazamox, fluridone and glyphosate on Delta Smelt (*Hypomesus transpacificus*). Aquatic Toxicology 197, 79–88. doi:10.1016/j.aquatox.2018.01.019.

[37] Kreutz, L.C., Barcellos, L.J.G., Marteninghe, A., dos Santos, E.D., Zanatta, R., 2010. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to Aeromonas hydrophila challenge. Fish & Shellfish Immunology 29, 694–697. doi:10.1016/j.fsi.2010.06.003.

[38] Lamb, S.D., Chia, J.H.Z., Johnson, S.L., 2020. Paternal exposure to a common herbicide alters the behavior and serotonergic system of zebrafish offspring. PLOS ONE 15, 1–22. doi:10.1371/journal.pone.0228357.

[39] Lanzarin, G.A., Félix, L.M., Santos, D., Venâncio, C.A., Monteiro, S.M., 2019. Dose-dependent effects of a glyphosate commercial formulation - Roundup® UltraMax - on the early zebrafish embryogenesis. Chemosphere 223, 514–522. doi:10.1016/j.chemosphere.2019.02.071.

[40] Le Du-Carrée, J., Morin, T., Danion, M., 2021. Impact of chronic exposure of rainbow trout, *Oncorhynchus mykiss*, to low doses of glyphosate or glyphosate-based herbicides. Aquatic Toxicology 230, 105687. doi:10.1016/j.aquatox.2020.105687.

[41] Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquatic Toxicology 101, 13–30. doi:10.1016/j.aquatox.2010.10.006, arXiv:arXiv:1011.1669v3.

[42] Marchand, J., Tanguy, A., Charrier, G., Quiniou, L., Plee-Gauthier, E., Laroche, J., 2006. Molecular Identification and Expression of Differentially Regulated Genes of the European Flounder, *Platichthys flesus*, Submitted to Pesticide Exposure. Marine Biotechnology 8, 275–294. doi:10.1007/s10126-005-0099-3.

[43] Matozzo, V., Munari, M., Masiero, L., Finos, L., Marin, M.G., 2019. Ecotoxicological hazard of a mixture of glyphosate and aminomethylphosphonic acid to the mussel *Mytilus galloprovincialis* (Lamarck 1819). Scientific Reports 9, 14302. doi:10.1038/s41598-019-50607-0.

[44] Menéndez-Helman, R.J., Ferreyroa, G.V., dos Santos Afonso, M., Salibián, A., 2011. Glyphosate as an acetylcholinesterase inhibitor in *cnesterodon decemmaculatus*. Bulletin of Environment Contamination and Toxicology 88, 6–9. doi:10.1007/s00128-011-0423-8.

[45] Menezes, C.C., Fonseca, M.B., Loro, V.L., Santi, A., Cattaneo, R., Clasen, B., Pretto, A., Morsch, V.M., 2010. Roundup Effects on Oxidative Stress Parameters and Recovery Pattern of *Rhamdia quelen*. Archives of Environmental Contamination and Toxicology 60, 665–671. doi:10.1007/s00244-010-9574-6.

[46] Modesto, K.A., Martinez, C.B., 2010. Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere 81, 781–787. doi:10.1016/j.chemosphere.2010.07.005.

[47] Myers, J.P., Antoniou, M.N., Blumberg, B., Carroll, L., Colborn, T., Everett, L.G., Hansen, M., Landrigan, P.J., Lanphear, B.P., Mesnage, R., Vandenberg, L.N., vom Saal, F.S., Welshons, W.V., Benbrook, C.M., 2016. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environmental Health 15, 19. doi:10.1186/s12940-016-0117-0.

[48] Nikolakakis, S., Bossier, P., Kanlis, G., Dierckens, K., Adriaens, D., 2014. Protocol for quantitative shape analysis of deformities in early larval European seabass *Dicentrarchus labrax*. Journal of Fish Biology 84, 206–224. doi:10.1111/jfb.12284.

[49] Norin, T., Malte, H., 2012. Intraspecific Variation in Aerobic Metabolic Rate of Fish: Relations with Organ Size and Enzyme Activity in Brown Trout. Physiological and Biochemical Zoology 85, 645–656. doi:10.1086/665982.

[50] OECD, 2006. Current approaches in the statistical analysis of ecotoxicity data: a guidance to application. OECD Environment Health and Safety Publications 54.

[51] Pédron, N., Du, J.L., Charrier, G., Zambonino-Infante, J.L., Bayon, N.L., Vasconcelos, R.P., Fonseca, V.F., Grand, F.L., Laroche, J., 2017. Contrasting patterns of energy metabolism in northern vs southern peripheral European flounder populations exposed to temperature rising and hypoxia. Marine Environment Research 129, 258–267. doi:10.1016/j.marenvres.2017.06.010.

[52] Pereira, A.G., Jaramillo, M.L., Remor, A.P., Latini, A., Davico, C.E., da Silva, M.L., Müller, Y.M., Ammar, D., Nazari, E.M., 2018. Low-concentration exposure to glyphosate-based herbicide modulates the complexes of the mitochondrial respiratory chain and induces mitochondrial hyperpolarization in the *Danio rerio* brain. Chemosphere 209, 353–362. doi:10.1016/j.chemosphere.2018.06.075.

[53] Persch, T.S.P., Weimer, R.N., Freitas, B.S., Oliveira, G.T., 2017. Metabolic parameters and oxidative balance in juvenile *Rhamdia quelen* exposed to rice paddy herbicides: Roundup®, Primoleo®, and Facet®. Chemosphere 174, 98–109. doi:10.1016/j.chemosphere.2017.01.092.

[54] Pimentel, M.S., Faleiro, F., Machado, J., Pousão-Ferreira, P., Rosa, R., 2019. Seabream Larval Physiology under Ocean Warming and Acidification. Fishes 5, 1. doi:10.3390/fishes5010001.

[55] R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria.

[56] Sánchez, J.A.A., Varela, A.S., Corcini, C.D., da Silva, J.C., Primel, E.G., Caldas, S., Klein, R.D., Martins, C.D.M.G., 2017. Effects of Roundup formulations on biochemical biomarkers and male sperm quality of the livebearing *Jenynsia multidentata*. Chemosphere 177, 200–210. doi:10.1016/j.chemosphere.2017.02.147.

[57] Seber, G., Wild, C., 1989. Growth Models. Wiley Series in Probability and Statistics, pp. 325–365. doi:10.1002/0471725315.ch7.

[58] Smith, C.M., Vera, M.K., Bhandari, R.K., 2019. Developmental and epigenetic effects of Roundup and glyphosate exposure on Japanese medaka (*Oryzias latipes*). Aquatic Toxicology 210, 215–226. doi:10.1016/j.aquatox.2019.03.005.

[59] Sobjak, T.M., Romão, S., do Nascimento, C.Z., dos Santos, A.F.P., Vogel, L., Guimarães, A.T.B., 2017. Assessment of the oxidative and neurotoxic effects of glyphosate pesticide on the larvae of *Rhamdia quelen* fish. Chemosphere 182, 267–275. doi:10.1016/j.chemosphere.2017.05.031.

[60] Stenz, L., Schechter, D.S., Serpa, S.R., Paoloni-Giacobino, A., 2018. Intergenerational Transmission of DNA Methylation Signatures Associated with Early Life Stress. Current Genomics 19, 665–675. doi:10.2174/1389202919666171229145656.

[61] Sulukan, E., Köktürk, M., Ceylan, H., Beydemir, Ş., Işik, M., Atamanalp, M., Ceyhun, S.B., 2017. An approach to clarify the effect mechanism of glyphosate on body malformations during embryonic development of zebrafish (*Danio rerio*). Chemosphere 180, 77–85. doi:10.1016/j.chemosphere.2017.04.018.

[62] Székács, A., Darvas, B., 2018. Re-registration Challenges of Glyphosate in the European Union. Frontiers in Environmental Science 6, 78. doi:10.3389/fenvs.2018.00078.

[63] Therneau, T.M., 2015. A Package for Survival Analysis in R.

[64] Topal, A., Atamanalp, M., Uçar, A., Oruç, E., Kocaman, E.M., Sulukan, E., Akdemir, F., Beydemir, Ş., Kılınç, N., Erdoğan, O., Ceyhun, S.B., 2015. Effects of glyphosate on juvenile rainbow trout (*oncorhynchus mykiss*): transcriptional and enzymatic analyses of antioxidant defence system, histopathological liver damage and swimming performance. Ecotoxicology and Environmental Safety 111, 206–214. doi:10.1016/j.ecoenv.2014.09.027.

[65] Torretta, V., Katsoyiannis, I., Viotti, P., Rada, E., 2018. Critical review of the effects of glyphosate exposure to the environment and humans through the food supply chain. Sustainability 10, 950. doi:10.3390/su10040950.

[66] Webster, T.M.U., Laing, L.V., Florance, H., Santos, E.M., 2014. Effects of Glyphosate and its Formu-

lation, Roundup, on Reproduction in Zebrafish (*Danio rerio*). Environmental Science & Technology 48, 1271–1279. doi:10.1021/es404258h.

[67] Webster, T.M.U., Santos, E.M., 2015. Global transcriptomic profiling demonstrates induction of oxidative stress and of compensatory cellular stress responses in brown trout exposed to glyphosate and Roundup. BMC Genomics 16, 32. doi:10.1186/s12864-015-1254-5.

[68] Weeks Santos, S., Cormier, B., Mazzella, N., Bonnaud, B., Morin, S., Clérandeau, C., Morin, B., Cachot, J., 2019. A glyphosate-based herbicide induces sub-lethal effects in early life stages and liver cell line of rainbow trout, *Oncorhynchus mykiss*. Aquatic Toxicology 216, 105291. doi:10.1016/j.aquatox.2019.105291.

[69] Welch, B.Y.B.L., 2012. Biometrika Trust. Biometrika 38, 330–336.

[70] Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York.

[71] Zebral, Y.D., Costa, P.G., de Castro Knopp, B., Lansini, L.R., Zafalon-Silva, B., Bianchini, A., Robaldo, R.B., 2017. Effects of a glyphosate-based herbicide in pejerrey *Odontesthes humensis* embryonic development. Chemosphere 185, 860–867. doi:10.1016/j.chemosphere.2017.07.069.

[72] Zhang, S., Xu, J., Kuang, X., Li, S., Li, X., Chen, D., Zhao, X., Feng, X., 2017. Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*). Chemosphere 181, 270–280. doi:10.1016/j.chemosphere.2017.04.094.