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Impact of chronic exposure of rainbow trout, *Oncorhynchus mykiss*, to low doses of glyphosate or glyphosate-based herbicides

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Abstract

Glyphosate is an herbicidal active substance (AS) entering in the composition of a large diversity of pesticide products (glyphosate-based herbicides; GBH) used in modern intensive agriculture. This compound has a favorable environmental safety profile but was suspected to induce deleterious effects in aquatic organisms, with a potential effect of some associated co-formulants. This study aimed to assess the impact of direct and chronic exposure to glyphosate on the health status of rainbow trout, *Oncorhynchus mykiss*. A total of 36 genitors were exposed daily for 10 months to a dose of glyphosate representative of environmental concentrations (around $1 \mu\text{g L}^{-1}$) using the AS alone or two GBHs formulations (i.e. Roundup Innovert[®] and Viaglif Jardin[®]) and findings were compared to an unexposed control group (n = 12). The effects of chemical exposure on the reproductive capacities, hemato-immunologic functions, energetic metabolism, oxidative stress and specific biomarkers of exposure were analyzed over a period of 4 months covering spawning. A limited mortality between 15 and 30% specific to the spawning occurred under all conditions. No differences were observed in reproduction parameters i.e. mean weights, relative fertility and fecundity. Red blood cell count, hematocrit index, mean corpuscular volume and white blood cell counts were similar for all the sampling dates. Significant changes were observed two months before spawning with a 70% decrease of the proportion of macrophages in trout exposed to Viaglif only and a reduction of 35% of the phagocytic activity in fish exposed to the two GBHs. Trends towards lower levels of expression of tumor necrosis factor- α (between 38 and 66%) were detected one month after the spawning for all contaminated conditions but without being statistically significant. Biomarkers of exposure, i.e. acetylcholine esterase and carbonic anhydrase activities, were not impacted and none of the chemical contaminants disturbed the oxidative stress or metabolism parameters measured. These results suggest that a 10 months exposure of rainbow trout to a concentration of $1 \mu\text{g L}^{-1}$ of glyphosate administered using the pure active substance or two GBHs did not significantly modify their global health including during the spawning period. The immunological disturbances observed will need to be further explored because they could have a major impact in response to infectious stress.

Highlights

- Exposure to glyphosate alone or co-formulated was studied in rainbow trout
- Chronic low doses representative of environmental levels were studied for 10 months
- No major changes in reproduction, metabolism nor oxidative response were observed
- Occasional impacts of glyphosate-based herbicides on immune response occurred

Keywords: Glyphosate, Glyphosate-based herbicide, Ecotoxicity, reproduction, Immune system

1. Introduction

In the context of rapid human population growth, a major challenge for agriculture is to meet food production demand by using sustainable practices that protect both human health and the environment [80]. Modern intensive agriculture is economically highly dependent on the use of agrochemical products [83]. Among the broad range of substances available on the market, glyphosate is one of the most widely used herbicide compounds in the world. Its massive use, enhanced by the development of genetically modified glyphosate-resistant crops [79, 82], has resulted in high prevalence of the compound in the environment. Glyphosate is found in different environmental compartments, including surface water. Although higher concentrations are detected in countries where farming of glyphosate-resistant crops is authorized (e.g. levels from 2 to 430 $\mu\text{g L}^{-1}$ in United states in stream and river water), glyphosate is present in water sources in Europe where levels above regulatory limits have been observed [86]. In France, between 2007 and 2017, glyphosate and aminomethyl phosphonic acid (AMPA, the first metabolite of its degradation pathway) have been detected in 43.1 to 74.4% of environmental samples with mean maximal annual concentrations ranging from 2.4 to 70.2 $\mu\text{g L}^{-1}$ and 8.4 to 67.8 $\mu\text{g L}^{-1}$, respectively [2]. The ubiquity of this herbicide compound has raised particular concern for its effects on human health and the environment.

The physico-chemical properties of glyphosate associated with its specific action on 5-enolpyruvoylshikimate 3-phosphate synthase, an enzyme present exclusively in plants and some micro-organisms, suggest a favorable environmental safety profile [30]. In a report of the European Food Safety Authority (EFSA) [25], half maximal effective concentration (EC50) of 38 and 306 mg L^{-1} were reported for rainbow trout (RT) exposed to glyphosate and a commercial formulation, respectively, considering mortality during an exposure of 96 h. Reported value of chronic toxicity, for RT exposed during 85 days to glyphosate, was a no observed effect concentration (NOEC), based on growth, of 9.6 mg L^{-1} [25]. As a

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result, EFSA has classified the impact of glyphosate on aquatic organisms as "limited" [25]. However, the number of studies reporting deleterious effects of this herbicide on aquatic organisms has increased, and a strong need to focus on environmentally relevant chronic low dose exposures have been pointed out (reviewed by Annett et al. [3]). In addition, there has been considerable debate in the context of the re-registration of glyphosate in the European Union (EU) concerning the potential toxic effects of the active substance (AS), but also of the co-formulants integrated into finished products called glyphosate-based herbicides (GBHs) to increase efficacy. Some co-formulants have already been shown to be more toxic than the AS and synergistic interactions among the different chemicals present in formulations may occur [3, 79].

Pesticides can act at different levels of biological organization [61]. At the molecular level, inhibition of carbonic anhydrase (CA), a key respiration enzyme involved in blood transport of CO₂, has been demonstrated to be a target of chemical contaminants ([10, 39, 53, 78]; reviewed by Lionetto et al. [44]). Sulukan et al. [78] showed that inhibition of this enzyme could be associated with respiratory acidosis in zebrafish (*Danio rerio*) larvae exposed to a GBH with a concentration of glyphosate comprised between 1 to 100 mg L⁻¹, resulting in adverse effects such as apoptosis and malformations during development. Pure glyphosate has a capacity to inhibit acetylcholine esterase (AChE) activity *in vitro*, however only at relatively high concentrations [21]. Glyphosate, associated or not to co-formulant, have been shown to inhibit fish AChE at concentration ranging from 0.2 to 20 mg L⁻¹ for GBH [9, 56, 31, 33] and from 1 to 30 mg L⁻¹ for AS [54]. Muscle hyperactivity induced by AChE inhibition could generate oxidative radicals inducing oxidative damage such as lipid peroxidation [9, 56]. Oxidative stress is known to be activated by environmental contaminants such as pesticides, through direct and indirect mechanisms [48]. Furthermore, several studies have pointed out that the toxic mode of action of glyphosate alone or GBHs in non-targeted species might be directly or indirectly associated with the generation of oxidative stress [47, 61, 9, 78].

These types of changes in the equilibrium of cellular physiology could have adverse effects at higher levels of biological organization. In particular, energetic metabolism plays a key role in the tolerance of an organism to environmental stressors [76]. Several studies indicate that glyphosate AS and/or GBHs could impact the metabolism of fish [43, 77, 74, 51, 65, 31, 85, 33]. Particularly, Topal et al. [81] observed a reduction of swimming performance possibly due to the dysregulation of energy metabolism in rainbow trout exposed short or long term to a GBH at concentrations of glyphosate comprised between 2.5 to 10 mg L⁻¹. Disruptions of energy production and key metabolic pathways could result in a trade-off in the allocation of energy used for fitness dependent functions such as growth, survival, and reproduction [76]. Therefore, the impact of glyphosate on reproduction, using fecundity and fertility indices, is of particular interest. More specific processes, such as the steroidogenic biosynthesis pathway, can also be altered as suggested for zebrafish [84].

Innate and acquired immunity play a crucial role in the survival of individuals facing infectious diseases. Several hematologic and immunologic blood parameters were shown to be altered [49, 50, 41] in fish exposed to glyphosate, potentially resulting in higher susceptibility to bacterial infections [42], but the immunotoxicity potential of this AS is still poorly defined.

The present study investigated the impact of a chronic exposure to environmentally

relevant low doses of glyphosate on the overall health of genitors of rainbow trout *O. mykiss*, a species particularly sensitive to several aquatic contaminants and commonly produced in Europe for food consumption [23, 60]. Toxicity of the AS was compared to that of two GBHs (Roundup Innovert[®] and Viaglif Jardin[®]) across different levels of organization including reproduction, immunity, and antioxidant defense systems.

2. Materials and methods

2.1. Ethics Statement

All animal studies were carried out in strict accordance with the European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). All animal experimental procedures were approved by the ethics committee on animal experimentation ANSES/ENVA/UPC No. 16 and were authorized by the French Ministry of Research, under the number APAFIS#2017090117104091. Blood sampling and stripping were done on fish anesthetized with 2% eugenol (4-allyl-2-methoxyphenol). Euthanasia involved addition of a lethal dose of 100 ppm of Eugenol into tank water. Compassionate euthanasia was planned for animals showing lesions (damaged fins, wounds) or abnormal behavior during the experiment.

2.2. Fish maintenance

Forty-eight specific pathogen free (SPF) rainbow trout including a mixture of three-year-old males and females from the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France) were used. Fish were placed in four 400 L tanks. All tanks have been grouped in the same room and maintained identically to limit inter-tank variability as much as possible. Fish were maintained under continuous flow-through conditions (300 L h⁻¹) and oxygen levels were maintained above 60% of saturation with aeration. Fish were fed daily with appropriate food at 1.5% of the biomass. Fish were submitted to the natural photoperiod occurring in Brest (France) throughout the time of the experiment and a temperature increasing from 9 to 21 °C between April and July and decreasing from 21 to 8 °C between July and November.

2.3. Chemical exposure

Four conditions of contamination were tested, each including 12 RT (proportion of 1 male for 2 females): control (C; non-exposed RT), glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6), Roundup Innovert[®] (R; Agrilisa - for professional use) and Viaglif Jardin[®] (V; Agrilisa - for home gardens). G had a purity of 98%, while the concentration of R and V were 360 and 420 g L⁻¹ of glyphosate, respectively. The nature and concentrations of the co-formulants of the two commercial products, R and V, are unknown. Concentrated solutions of each product (4 mg L⁻¹) were prepared and stored under appropriate conditions (darkness, controlled temperature). From January to November 2018 (i.e. 10 months), every working day (generally 5 days a week), a volume of 100 mL of each of these respective solutions was added to the tanks hosting the fish for which the freshwater flow was stopped for one hour. After one hour of contact, regulated water flow was set up for the rest of the

day at 135 L h^{-1} , allowing for the gradual dilution of glyphosate. Theoretical kinetics of glyphosate concentrations in contaminated tanks are presented in Figure 1 and have been calculated from the equation 1. The integrated mean theoretical concentration of glyphosate for each contamination day was approximately 123 ng L^{-1} (the area integrated is represented by a blue zone on the theoretical dilution curve in Figure 1).

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

2.4. Sampling date and spawning of RT

2.4.1. Water sampling

Two sampling dates were performed in February and November, respectively at the beginning and at the end of the exposure period, to measure glyphosate concentrations in water tanks. The first samples were analyzed using the ELISA method and the second by HPLC. For each sampling date, 150 mL water samples were taken in each tank using sterile glass bottles. They were stored at -20°C until the ELISA test or directly sent, at 4°C , to the external provider for HPLC analysis.

2.4.2. Fish sampling

Non-invasive sampling was carried out two and one months before spawning (2mbs and 1mbs) by withdrawing 2mL of blood from the caudal vein with a lithium heparin vacutainer (Greiner ref. KG454244). For flow cytometry analyses, 300 μL of blood collected from each fish were immediately diluted with 10 mL of Leibovitz 15 medium (L15, Eurobio) containing 8 mg L^{-1} of lithium heparin (Sigma-Aldrich ref. H0878). Whole blood was used for hematologic analyses, while the remaining blood samples were centrifuged ($1,200 \times g$, 10 min, 4°C) and plasma was stored at -80°C for immunological analysis. One month after spawning (1mas), another blood sample was taken and fish were euthanized. Then, the liver, spleen, gills and brain were sampled, flash-frozen in liquid nitrogen and stored at -80°C for future analyses.

2.4.3. Spawning

The spawning period occurred in November 2018 and the maturation of fish was tested every week by exerting manual abdominal stripping to ensure spawning on a day when most of the fish were mature. Stripping events were done twice over the spawning period to collect eggs and sperm from the most mature fish, with approximately two weeks between them. For each stripping event, sperm of all mature males were used to fertilize eggs of mature females belonging to the same experimental condition. After the stripping, females were weighed before and after stripping to measure the total weight of eggs. A fraction of eggs for each mature female was sampled in modified Gilson's solution (as described by Friedland et al. [26], without nitric acid) for egg numbering and weighing. Relative fecundity was considered as the number of eggs divided by the weight of each respective female after stripping [11].

2.4.4. *Survival of the eggs*

Eggs of each female were independently fertilized with the pool of sperm collected for each respective chemical condition. Embryonic development was conducted in tank, containing approximately 300 L, continuously renewed with a river water flow (approximately 300 L h^{-1}). Temperature during embryonic development was maintained at $8^\circ\text{C} \pm 2^\circ\text{C}$. Water quality parameters were monitored as described for adult fish. Fertility was considered to be the proportion of eggs surviving at five days post fertilization [11]. To perform this measurement, 4, 7, 7 and 5 females were considered for the C, G, R and V respectively. Survival of eggs was assessed daily for each female on a fraction of approximately 200 eggs isolated in plastic breeding boxes.

2.5. *Chemical analysis*

Quantification of glyphosate using direct competitive ELISA assay (Novakits, ref. 1500086) was done at two times of the daily kinetics (i.e. just before the water flow reopening, wfr, and 2 hours after wfr) in all tanks, following supplier's instructions. Briefly, 50 μL of river water was filtered and derivatized, before to be transferred into wells of a microplate coated with goat anti-rabbit antibodies. Sample was diluted at 1/2 with rabbit anti-Glyphosate antibody reagent and competitive reaction with enzyme conjugate solution lasted 1 h. After a step of washing, the colored reaction was started by adding 150 μL of substrate solution to the wells. The reaction was stopped after 25min, and plates were read at 450 nm on a TECAN Spark 10M microplate spectrophotometer. The development of the color is inversely proportional to the concentration of AS in the sample. Standard curve has been realized using the four-parameter log-logistic function, LL.4, of the R package "drc" [75]. Quantification was done by reporting the OD value obtained for the sample to the quantification standard curve obtained for each assay.

Quantification of glyphosate and AMPA by HPLC/fluorometric methods (Method ref. ANA-I10.MOA.69.B) was done by an external provider (Laboceca, France) at different times of the daily kinetics (i.e. just before wfr and 1, 2 and 3 hours after wfr), in the tank contaminated with the AS.

2.6. *Hematologic and immunologic parameters*

The hematocrit index was measured using microhematocrit capillary tubes after 5 minutes centrifugation at $12,000 \times g$. Counting of red and white blood cells was performed on a Thoma cell hemocytometer using whole blood diluted to 1/200 in Giemsa solution [38]. Mean corpuscular volume (MCV) was expressed as the ratio of the hematocrit index to the number of erythrocytes per unit of volume. Blood leukocyte populations were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson). Leukocytes were extracted on a Ficoll gradient (Histopaque[®] 1077, Eurobio) after several centrifugation cycles according to Danion et al. [15]. For each sample, cell parameters were analyzed on 15,000 events. Two leukocyte populations, lymphocytes and macrophages, were differentiated according to their scatter properties. Phagocytic activity was measured with a solution of leukocytes with 2.7×10^9 fluorescencebeads/mL (FluoroSpheres[®] carboxylate-modified microspheres, diameter 1 μm , molecular probes, ThermoFisher Scientific) after 1 hour of

incubation at room temperature. Phagocytic capacity was considered to be the proportion of leukocytes that presented a fluorescence intensity equal to or greater than two beads (modified from Brousseau [7]). Note that due to a technical problem, phagocytic activity could not be obtained at 1mbs. The alternative pathway of plasma complement activity was measured according to Danion et al. [16]. Results were expressed using the formula described by Costabile [13].

2.7. Gene expression analysis

Total RNA was isolated using an RNeasy Plus kit (Qiagen) from 20 mg of spleen sampled at 1mas, and all samples were treated with the RTS DNase Kit (Mobio) to prevent DNA contamination. For each sample, RT was carried out using 1 µg total RNA with a RevertAid H minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and an iCycler System (Bio-Rad). mRNA was quantified for the following four genes: complement (*C3-1* and *C3-4*), interleukin-1β (*IL-1β*) and tumor necrosis factor-α (*TNF-α*). In addition, the elongation factor gene *EF* was included in the analysis as a housekeeping gene. Primers were obtained from the literature and are based on sequences available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>; Table 1). The characteristics of the quantitative real-time PCR (qPCR) reactions performed in the CFX96 real-time PCR System (Bio-Rad) are available in [20]. Relative expression of the target gene was calculated with the Pfaffl formula (equation 2; [69]). The ratio of a target gene was expressed in a sample versus a control, in comparison to a reference gene.

$$\frac{\text{Efficacy of target gene}^{Ct_{target\ pool} - Ct_{target\ sample}}}{\text{Efficacy of } EF \text{ gene}^{Ct_{ef\ pool} - Ct_{EF\ sample}}} \quad (2)$$

Table 1: Immune genes targeted and primers used in this study

Gene	Primer.sequences (5' to 3')	Genbank.references	Source	Efficiencias
<i>EF</i>	Forward: GATCCAGAAGGAGGTCACCA Reverse: TTACGTTTCGACCTTCCATCC	AF498320.1	Ballesteros et al. [4]	1.12
<i>C3-1</i>	Forward: GAGATGGCCTCCAAGAAGATAGAA Reverse: ACCGCATGTACGCATCATCA	L24433.1	Cortés et al. [12]	1.19
<i>C3-4</i>	Forward: TGCTCTAGTCACATCTACCACTCT Reverse: CAGGCACATGGGTATAACAGTAGAA	AF271080	Løvoll et al. [45]	1.18
<i>Il-1</i>	Forward: ACCGAGTTCAAGGACAAGGA Reverse: CATTCATCAGGACCCAGCAC	DQ778946	Bjork et al. [5]	1.18
<i>TNF-α</i>	Forward: GGGGACAAACTGTGGACTGA Reverse: GAAGTTCTTGCCCTGCTCTG	AJ277604	Pérez-Sánchez et al. [67]	1.20

2.8. Bio-markers, metabolic parameters and oxidative stress

All organs were homogenized 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). Protein concentrations in the extracts were measured using the Pierce BCA protein assay kit (ThermoFisher Scientific). Each

assay measurement was performed in triplicate. All enzymatic reactions were monitored using a TECAN Spark 10M microplate spectrophotometer. Metabolic parameters were calculated using the slope of the curve $optical\ density = f(time)$ and the Beer-Lambert law ($A = \epsilon lc$). Oxidative stress activities were calculated using calibration curves generated using standards. All results were expressed in specific activities (IU mg^{-1}).

. Choline esterases (ChE) were measured in brain following a protocol adapted from Ellman et al. [22]. Briefly, 20 μ L of extract were assayed in 140 μ L of a reaction solution with acetylthiocholine iodide and dithiobis-2-nitrobenzoic acid. The variation in optical density was recorded at 412 nm.

. Carbonic anhydrase (CA) was assayed in gill following a protocol adapted from Verpoorte et al. [87] using a reaction solution with 4-nitrophenyl acetate and acetazolamide (AZM) or dimethyl sulfoxide (DMSO) (total esterase activity without or with CA activity, respectively). The enzymatic reaction was monitored spectrophotometrically at 405 nm.

. Citrate synthase (CS), Cytochrome c oxidase (CCO), Lactate dehydrogenase (LDH) and Glucose-6-phosphate dehydrogenase (G6PDH) metabolic parameters were measured in liver at, respectively, 412 nm, 550 nm, 340 nm and 340 nm, following an adaptation of the procedures described by Gauthier et al. [28].

. Thiobarbituric acid reactive substances (TBARS) were quantified by their reactivity with thiobarbituric acid in acidic conditions according to Espín et al. [24].

. Total activity of superoxide dismutase (totalSOD) and manganese-dependent superoxide dismutase (MnSOD) activity were measured in liver according to Espín et al. [24], Mesa-Herrera et al. [55]. Briefly, samples were assayed in a solution of pyrogallol and the rate of pyrogallol autoxidation was measured with a spectrophotometer at 325 nm. MnSOD activity was calculated by substrating the activity of totalSOD by the activity of SOD measured in presence of an inhibitor of MnSOD, the sodium cyanide

. Gluthathione Peroxidase (GPx) activity was determined in liver using 15 μ L of diluted sample according to the Paglia method [63]. Cumene hydroperoxide was used as the substrate and enzymatic activity was assessed at 340 nm.

. Catalase (CAT) was assayed in liver using the method of Aebi [1]. Briefly, the assay mixture consisted of phosphate buffer (100 mM, pH 7) and H₂O₂ (28 mM). The variation of H₂O₂ absorbance was measured with a spectrophotometer at 225 nm.

2.9. Data processing and statistical analyses

Data processing and statistical analyses were conducted with R software [71], except for normalized gene expressions that were obtained with Bio-RAD CFX Manager™ Software (Bio-Rad). Figures were produced using the ggplot2 package [89]. All the data were tested for normality and homoscedasticity. In the case of normal and homoscedastic data, one-way Anova tests were used to compare means, followed by a post-hoc test of Dunnett [19]. In the

case of normal and heteroscedastic data, modified one-way Anova tests were used to compare means [88], followed by a post-hoc test of Tamhane-Dunnett [59]. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn [18]. Differences between survival and fecundity percentages were compared with a Chi-squared test. A p-value of 0.05 was used as the threshold for statistical significance. A test of correlations between variables was carried out using the `lmrob` R function (R package `robustbase`; Wilcox [90]).

3. Results

3.1. Concentration assays

Glyphosate and AMPA were not detected in control tank throughout the experiment. AMPA was never detected in any of the exposure tanks. Using ELISA assay, concentrations of glyphosate ranged between 1.05 and 1.45 $\mu\text{g L}^{-1}$ in all exposure tanks one hour after the addition of the chemical (Figure 1). The glyphosate concentration measured by HPLC was approximately 30 to 40% lower (0.77 $\mu\text{g L}^{-1}$) at the same point of the kinetic. Two hours after restarting the water flow, glyphosate concentrations were similar (between 0.24 and 0.55 instead of the modeled concentration of 0.51 $\mu\text{g L}^{-1}$ predicted by the theoretical dilution curve) regardless of the method used.

3.2. Survival and growth parameters

No mortality was recorded before the spawning period. However, spawning induced mortality rates between 15 and 30 % (data not shown), without significant differences between the exposure conditions, that were randomly distributed across all treatment groups.

Mean ratios of weights before and after the spawning period were 1.14, 1.11 and 1.15 for control, glyphosate and Viaglif, respectively, while a ratio of 1.03 was found for the Roundup condition. No statistical differences were found among the means of the four exposure conditions.

3.3. Reproductive parameters

High fertility was observed for RT in all conditions. Relative fecundity and fertility between control and contaminated fish were not significantly different (Table 2).

3.4. Hematologic and immune blood parameters

No statistically significant change was observed among hematologic parameters (i.e. RBCC, HT, MCV and WBCC) of adult trout exposed to glyphosate or GBHs compared to controls (Table 3) before, during and after the spawning period (Table 3).

Considering the proportion of macrophages (Figure 2), the only statistically significant change was a reduction of approximately 70% for Viaglif condition ($\chi^2 = 12.2$; degrees of freedom, $df = 3$; p-value = 0.007) compared to the control two months before the spawning. At the same period, phagocytic capacity (Table 3) was significantly lower for fish exposed to GBHs compared to controls ($\chi^2 = 19.7$; $df = 3$; p-value = 0.0002). ACH₅₀ activity was not significantly altered in adult trout exposed to glyphosate or GBHs (Table 3).

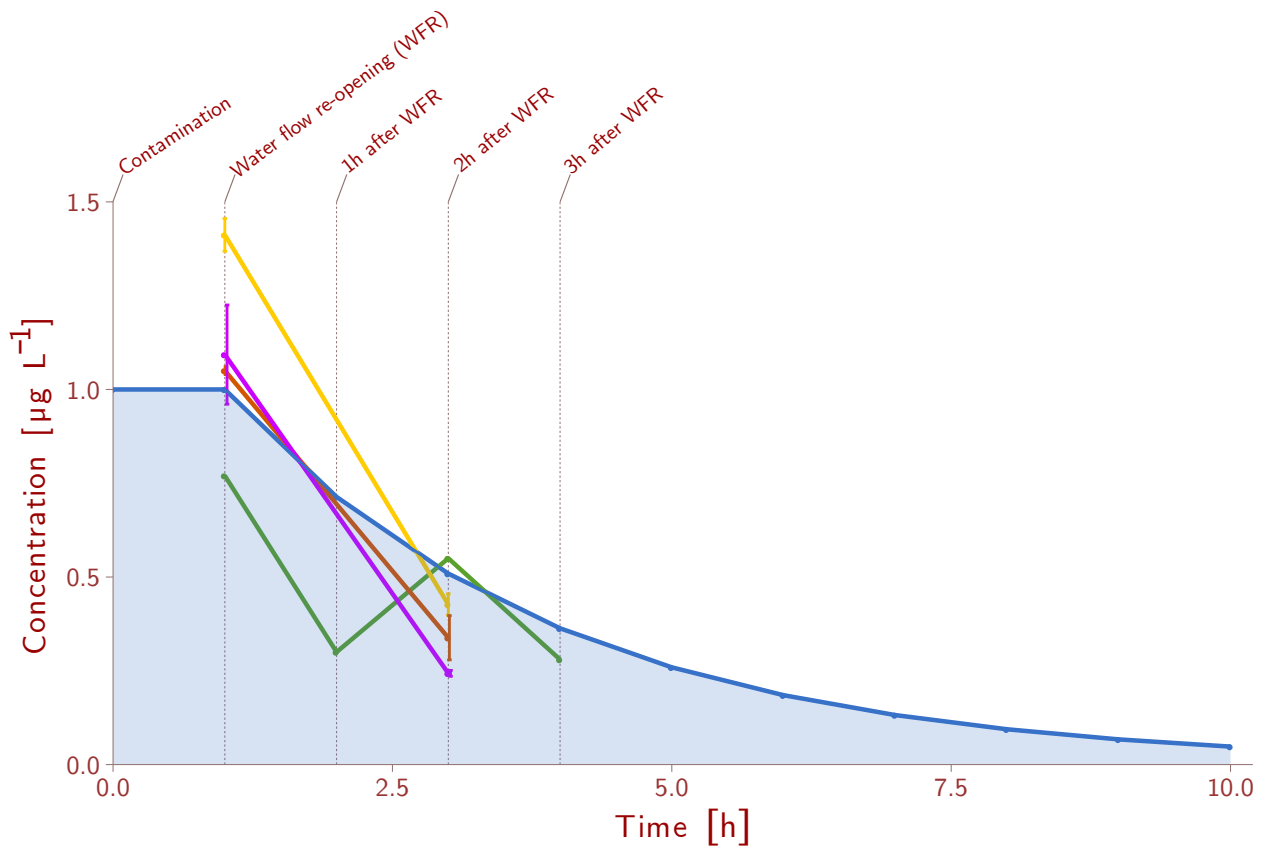


Figure 1: Mean concentration of glyphosate as a function of time ($\mu\text{g L}^{-1}$). Theoretical concentrations (in blue) are compared to concentrations measured at different times of the kinetic using ELISA or HPLC methods. ELISA analysis was done after 1 month of contamination for each condition (Glyphosate - G in yellow, Roundup - R in orange and Viaglif - V in purple) and two points of the kinetics. HPLC analysis was done after 10 months of contamination in the tank contaminated with G for all points of the kinetics (in black). Error bars represent standard-errors

Table 2: Mean reproductive parameters (\pm standard-error) of female RT. Relative fecundity corresponds to the ratio of the number of eggs to the weight of the fish (eggs g^{-1}). Fecundity is the proportion of surviving eggs five days post fertilization (%). n is comprised between 4 and 8 for relative fecundity and between 4 and 7 for fertility

Condition	Reproductive parameters	
	Relative fecundity (eggs g^{-1})	Fertility (%)
C	2.63 ± 0.72	99.6 ± 0.4
G	2.71 ± 0.30	95.8 ± 3.9
R	2.55 ± 0.22	99.8 ± 0.1
V	2.47 ± 0.32	99.5 ± 0.5

Table 3: Immuno-hematologic parameters measured in blood of contaminated RT in the period covering the spawning. Parameters indicated were analyzed for contaminated (G, R, V) and control (C) fish at 1 and 2 months before (mbs), during or 1 month after (mas) spawning. Values are expressed as means with standard-error. RBCC: Red blood cell count ($12 \geq n \geq 8$); HT: Hematocrit index ($11 \geq n \geq 8$); MCV: Mean corpuscular volume ($11 \geq n \geq 8$); WBCC: White blood cell count ($12 > n > 8$); PC: Phagocytic capacity ($12 \geq n \geq 7$); ACH₅₀: 50% Alternative haemolytic complement activity ($12 \geq n \geq 6$). The numbers in bold with an asterisk are significantly different from the values obtained for the control condition at the same period. ND : not done

Date	Condition	RBCC (TL)	HT (%)	MCV (fL)	WBCC (GL)	PC (%)	ACH ₅₀ (CH ₅₀ units mL ⁻¹)
1 mbs	C	1.72 (0.096)	ND	ND	31.33 (3.83)	ND	19.58 (1.54)
	G	1.98 (0.158)	ND	ND	46.33 (5.6)	ND	23.06 (1.02)
	R	2.02 (0.165)	ND	ND	34.73 (2.97)	ND	20.94 (1.34)
	V	1.98 (0.175)	ND	ND	46.33 (7.45)	ND	19.47 (2.17)

2 mbs	C	1.3 (0.12)	ND	ND	38.6 (2.86)	46.36 (3.63)	20.76 (1.34)
	G	1.27 (0.094)	ND	ND	38.5 (2.4)	38.7 (2.63)	21.17 (1.36)
	R	1.22 (0.074)	ND	ND	37.82 (4.03)	29.34* (2.57)	18.95 (1.6)
	V	1.34	ND	ND	41.17	28.59*	19.84

Continued on next page

Table 3 – continued from previous page

Date	Condition	RBCC (TL) (0.083)	HT (%) (1.67)	MCV (fL) (9.32)	WBCC (GL) (2.78)	PC (%) (2.15)	ACH ₅₀ (CH ₅₀ units mL ⁻¹) (2.01)
spawning	C	1.77 (0.168)	54.33 (1.67)	95.77 (9.32)	20.44 (3.98)	ND	17.93 (1.92)
	G	1.59 (0.146)	51.91 (3.61)	84.61 (10.44)	24.55 (4.86)	ND	20.82 (2.02)
	R	1.94 (0.089)	53 (2.37)	103.18 (6.94)	17.09 (1.65)	ND	16.68 (1.81)
	V	1.65 (0.186)	43.25 (4.42)	75.92 (13.83)	14.5 (1.64)	ND	17.49 (2.09)
1 mas	C	ND	ND	ND	ND	24.32 (3.85)	ND
	G	ND	ND	ND	ND	29.08 (2.83)	ND
	R	ND	ND	ND	ND	25.37 (3.45)	ND
	V	ND	ND	ND	ND	24.61 (3.5)	ND

RBCC: Red blood cell count ($12 > n > 8$); HT: Hematocrit index ($11 > n > 8$); MCV: Mean corpuscular volume ($11 > n > 8$); WBCC: White blood cell count ($12 > n > 8$); Macrophages ($12 > n > 7$); PC: Phagocytic capacity ($12 > n > 7$); ACH₅₀: 50% Alternative haemolytic complement activity ($12 > n > 6$)

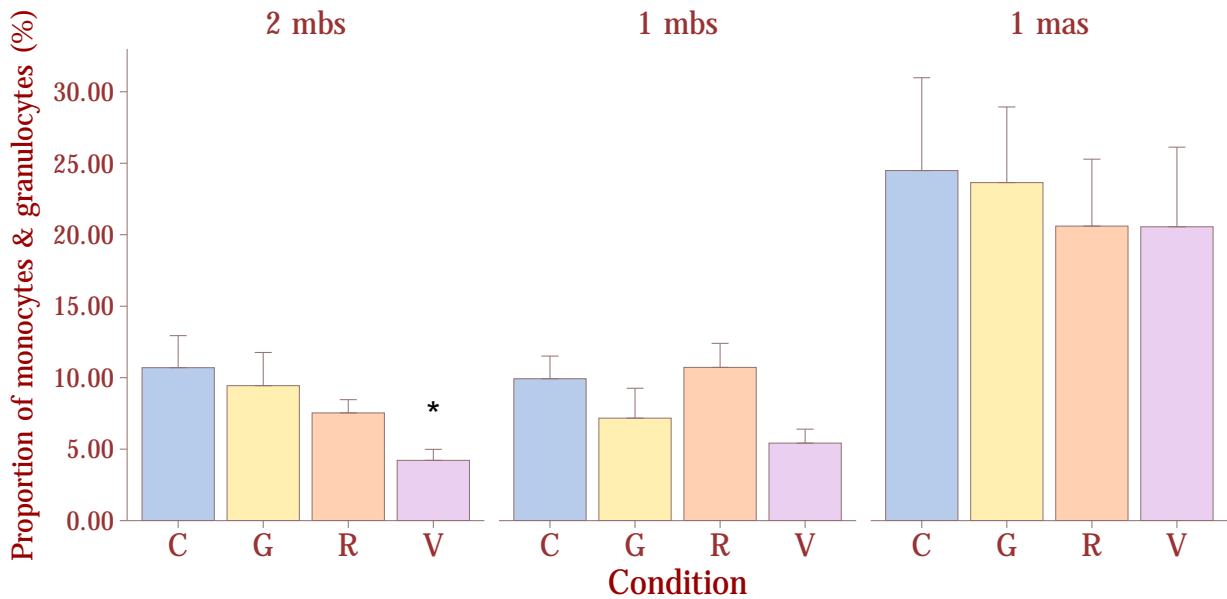


Figure 2: Mean proportions of macrophages and granulocytes in leukocyte populations of RT over the period covering spawning as a function of exposure conditions. Each window corresponds to a sampling time with mbs.= months before spawning; mas.= months after spawning. Standard errors are represented at the top of each bar ($12 \geq n \geq 7$). Each treatment was compared to the control (at each sampling time points). Statistically significant differences from control means are indicated with an asterisk ($p < 0.05$).

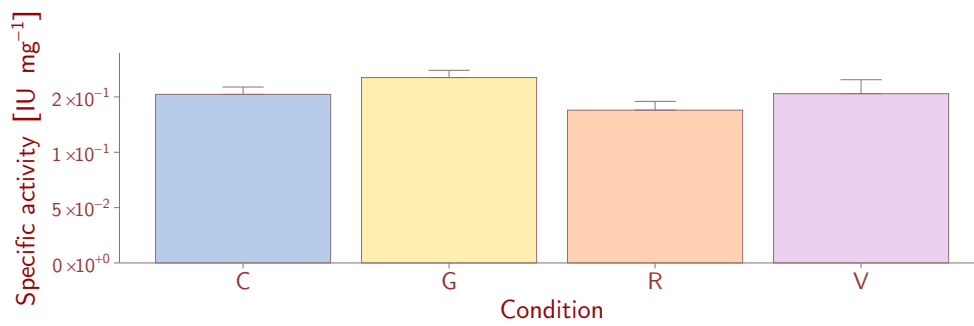
3.5. Immune gene expression in spleen

No significant differences between mean expressions were found one month after spawning for *C3-1*, *C3-4* and *IL1 - β* . An overall significant difference of means was observed for *TNF- α* expression ($f = 19.7$; $df = 3$; p -value = 0.045; Table 4), with a decrease of 38, 56 and 66% in the glyphosate, Roundup and Viaglif groups, respectively, compared to the control. A post-hoc test revealed no significant difference for *TNF- α* between chemically exposed conditions and control. A positive correlation was observed between the proportion of macrophages and the expression of *IL1 - β* (adjusted R-squared: 0.1571, $p = 0.001$) but not with *TNF- α* (adjusted R-squared: -0.02838, $p = 0.46$).

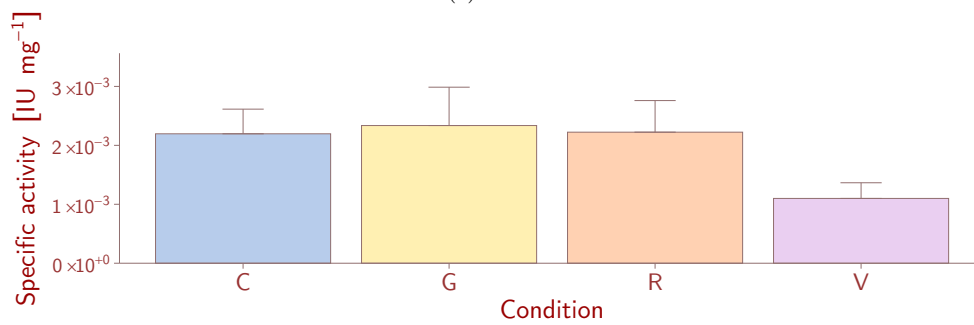
3.6. Biochemical parameters and oxidative stress in liver

No significant difference was noted in metabolic activities between contaminated and control fish one month after spawning (Table 5). Means of CS/CCO ratio values were 1.4 ± 0.07 , 1.3 ± 0.08 , 1.1 ± 0.05 and 1.2 ± 0.11 for control, glyphosate, Roundup and Viaglif conditions, respectively. For LDH/CS ratio, they were of 15.5 ± 0.38 , 17.3 ± 0.42 , 18.6 ± 0.95 and 16.9 ± 0.93 respectively. No significant difference was noted in any of these metabolic ratios between contaminated and control trouts.

No significant modification of the AChE and CA activities was observed one month after spawning in the brain and the gills of adult trout, respectively (Figure 3).



(a) AChE



(b) CA

Figure 3: Mean AChE and CA activities as a function of exposure conditions one month after spawning. Activities are expressed in (IU/mg of protein) for each contaminated conditions (G, R, V) and the control group (C) with standard errors represented at the top of each bar ($10 > n > 7$). AChE was measured in the brain and CA in the gills.

Table 4: Mean relative expression (\pm standard-error; log) of immune genes in the spleen one month after the spawning period. Results are expressed in Log and include standard errors.

Parameter	Condition of chemical exposure			
	C	G	R	V
<i>C3-1</i>	0.99 ± 0.57	1.86 ± 1.57	0.18 ± 0.11	1.39 ± 1.16
<i>C3-4</i>	0.6 ± 0.25	0.82 ± 0.54	0.2 ± 0.1	2.49 ± 2.27
<i>IL1-β</i>	1.71 ± 0.67	1.17 ± 0.35	0.71 ± 0.2	0.47 ± 0.09
<i>TNF-α</i>	1.21 ± 0.39	0.75 ± 0.11	0.54 ± 0.04	0.42 ± 0.06

n between 7 and 10

No significant difference was measured for MDA concentrations or CAT, GPx, SODmn, SODtotal and TBARS activities regardless of the chemical treatment occurred (Table 6) one month post-spawning.

1
2
3

Table 5: Mean of specific activities of enzymes involved in metabolic activities measured in the liver of contaminated RT one month after spawning. Data are expressed in IU mg⁻¹ of protein with standard error for each contaminated conditions (G, R, V) and the control group (C)

Parameter	Condition of chemical exposure			
	C	G	R	V
CS	0.02 (0.00082)	0.017 (0.0012)	0.017 (0.0008)	0.017 (0.00123)
CCO	0.014 (0.00079)	0.014 (0.00064)	0.015 (0.00094)	0.015 (0.00042)
LDH	0.3 (0.012)	0.29 (0.01)	0.31 (0.01)	0.29 (0.014)
G6PDH	0.053 (0.0081)	0.049 (0.0037)	0.048 (0.005)	0.052 (0.0061)

n between 7 and 10

Table 6: Means of specific activities and TBARS levels measured in livers of RT one month after spawning. Specific activities are expressed in (IU mg⁻¹ of protein) and TBARS levels as MDA concentrations in nmol mg⁻¹ of protein). Standard error are included in parenthesis.

Parameter	Condition of chemical exposure			
	C	G	R	V
CAT	10.14 (0.46)	10.85 (0.41)	11.81 (0.69)	11.43 (0.55)
GPx	0.38 (0.02)	0.41 (0.011)	0.39 (0.01)	0.36 (0.017)
SODmn	1.97 (0.18)	1.83 (0.24)	2.05 (0.21)	2.3 (0.29)
SODtotal	3.77 (0.24)	3.83 (0.37)	4.19 (0.37)	4.24 (0.42)
TBARS	0.018 (0.0023)	0.02 (0.0024)	0.022 (0.0033)	0.02 (0.003)

n between 6 and 10

4. Discussion

This study aimed to investigate the effect of long term exposure to low and environmentally relevant concentrations of glyphosate, the most widely used herbicide in the world, on the overall health status of rainbow trout, *Oncorhynchus mykiss*, a non-target species. In addition, another point of interest lay in the provision of data on the potential modulation of the toxicity of glyphosate by co-formulants of two commercial glyphosate-based herbicides (GBHs).

Our complex experimental design, studying chemical contamination over several months with a low environmentally relevant dose of glyphosate on sexually mature genitors, is particularly useful to understand the complexity of the toxic effects of this herbicide in the aquatic environment. In this study, only one tank per chemical treatment has been allocated to integrate the ethical rules of reducing the number of animals and the logistics constraints of keeping fish in good conditions throughout a prolonged period. To compensate the possible “tank effect”, we maximized the number of fish sampled to have a relevant overview of the individual variability occurring inside each tank in response to a specific contaminant. To minimize the inter-tank variability, the experimental conditions were strictly managed (i.e. all tanks were positioned in the same room and received the same water at the same temperature in open circuit). As these precautionary measures could not completely prevent the apparition of a “tank effect”, exposure reiteration will be necessary to confirm our results and hypothesis.

Levels of glyphosate contamination observed were relatively in phase with our theoretical kinetics, with a maximum variation of the expected concentration of glyphosate of approximately 23% for the HPLC method and 45% for the ELISA assay. The lower specificity of the ELISA compare to the HPLC method could explain the overestimation of glyphosate concentrations measured for several points of the kinetics. The first degradation metabolite of glyphosate, AMPA, was not detected in our contaminated tanks, probably because the water renewal prevented any accumulation of metabolites. No traces of glyphosate or AMPA were found in the control tank either, demonstrating that no external source of glyphosate was introduced into our experimental system. Glyphosate is known to have low bioconcentration factors in animal tissues [25, 37], and we therefore did not quantify the AS in fish. Nevertheless, a study of bio-markers of exposure has shown that the compound can be distributed at low doses in fish (reviewed by Annett et al. [3]).

Several authors have hypothesized that glyphosate, combined or not with co-formulants in GBHs, can cause toxicity through altering AChE and CA (AChE: [9, 56]; CA: [78]). AChE has been shown to be inhibited by concentrations of pure glyphosate of at least 1 mg L^{-1} and both enzymes were impacted by 0.2 mg L^{-1} and 1 mg L^{-1} of glyphosate formulated in several GBHs different from those tested here, respectively [9, 54, 56, 31]. Activation of AChE have also been reported during chronic exposure in freshwater stinging catfish (*Heteropneustes fossilis*) or in common carp (*Cyprinus carpio*) after a recovery period [17, 9] as well as hybrid situations coupling inhibition and activation, like in the mussel (*Mytilus galloprovincialis*) [52]. The development of a dynamic compensatory response in these aquatic organisms facing the stress induced by chemical exposure could explain the slight activation and inhibition of

AChE observed in the brains of our RT exposed to glyphosate and GBHs. Nevertheless, the dose of glyphosate exposure used was probably too low to induce changes in AChE activity. In vitro and in vivo inhibitions of CA have also been reported after exposure to a GBH, and it has been suggested that this inhibition could be responsible for other toxic effects at higher levels of biological organization [39, 78]. Although inhibition of the activity in animals contaminated with Viaglif seems to be emerging one month after spawning, no significant effect was measured in our study regardless of the condition. Complementary investigations will be necessary to confirm if this tendency lasts and grows with the exposure time.

Several enzymes implicated in both energy metabolism and antioxidant defenses have been monitored in livers of RT to assess the effect of chemical contamination in this organ that has been demonstrated to be the main target of glyphosate toxicity [47, 51]. Among them, malondialdehyde (MDA), an indicator of oxidative damage to lipids, was of particular interest as glyphosate and GBH have been described to induce oxidative stress and increase lipid peroxidation in fish [32, 58, 47, 61, 9, 78]. Most of the previous data on oxidative stress was obtained with glyphosate concentrations above 1 mg L^{-1} (i.e. a concentration 1000 times greater to the dose we used in the present study) and exposures were conducted with co-formulated products only. At lower concentrations, Persch et al. [68] no impact on TBARS levels was observed in different tissues of Catfish (*Rhamdia quelen*) juveniles exposed to concentrations of a GBH comprised between 18 and $144 \text{ } \mu\text{g L}^{-1}$ but reduction of glycogen and total lipid and protein contents occurred in this study. An increase of the CAT activity was also observed, particularly in gills at concentration above $36 \text{ } \mu\text{g L}^{-1}$. Under our conditions, we obtained only slight and no significant variations in activities of enzymes involved in defenses against oxidative stress. This is likely due to the fact that glyphosate concentrations used might be insufficient to induce an antioxidant response and damage in adult trouts. Previous authors had already reported that after a first phase of response with anti-oxidant enzymes induced in liver of the neotropical fish (*Prochilodus lineatus*) contaminated by a GBH, a subsequent phase occurred where enzymatic activities returned to baseline levels without an increase in oxidative damage [56]. Applied to our study, this means that adaptations occurring in the first phase of the chemical contamination might not be revealed by the pattern of oxidative stress defenses observed later in the experiment but also that, at the time of sampling, fish were protected from oxidative stress. G6PDH helps to protect against oxidative damage and is also recognized as being a relevant marker of anabolism [64]. G6PDH activity of our control group showed considerable interindividual variability, and the contaminants studied appeared not to influence the regeneration capacity of NADPH implicated in oxidative stress defenses, nor anabolic capacity. Several discrete disruptions were observed in chemically exposed fish for three liver enzymes that play key roles in metabolic pathways. LDH activity appeared not to be particularly influenced by chemical contamination, suggesting that fish did not increase anaerobic metabolism as a compensatory mechanism against loss of aerobic capacity [76]. Activities of enzymes involved in aerobic metabolism, CCO and CS, were not impacted by the chemical contamination. Some tendencies were observed when CS/CCO and LDH/CS ratios of activities were considered but further studies are needed to confirm these results. Higher LDH/CS could indicate a transition from aerobic to anaerobic metabolism [70], while

a lower CS/CCO ratio could reveal mitochondrial dysfunction [35]. Craig et al. [14] observed that Cu exposure affected this ratio in the liver of the gilthead seabream (*Sparus aurata*) and suggested that it could, therefore, affect normal mitochondrial biogenic processes. GBHs have been shown to generate mitochondrial dysfunction in isolated mitochondria [65], cells [46], and fish [66] and mitochondrial impairments were reported in the brain of zebrafish (*Daion rerio*) sub-chronically exposed to a GBH at concentration as low as $65 \mu\text{g L}^{-1}$ Pereira et al. [66]. Interestingly, mitochondrial effects and neurotoxicity did not seem to be linked to glyphosate or co-formulant concentrations.

Seasonal variations in hematologic parameters resulting in erythrocytosis or leukopenia are well documented in RT and explained by physiological adaptation to external conditions [57, 36] or to breeding cycles with gonad maturation [8]. Disturbance in hematologic parameters can also be induced by acute, sub-chronic or chronic exposure to GBHs, as reported in various fish species [41, 31, 29, 74, 56, 40]. We showed that hematological parameters had a tendency to fluctuate during the sexual maturation and the spawning period but chemical contamination with glyphosate or GBHs had no impact on these parameters. At the spawning time, RT contaminated with Viaglif showed a lower, but not significantly different from the control, hematocrit index associated with a lower MCV. The hematocrit value reflects the capacity of blood to transport oxygen and low hematocrit could have been associated with lower physiological performances [27]. The proportion of different leukocyte populations in fish exposed to GBHs presented a smaller proportion of macrophages and, consequently reduced phagocytic activity before the spawning period. These kinds of changes have already been reported after exposure to GBHs, and several authors have hypothesized that this could be the consequence of the oxidative stress induced by chemical contaminants which increases production of reactive oxygen species and damage cell membranes [62, 42, 41]. The present study does not allow the validation of this hypothesis and further investigations will be necessary to demonstrate the involvement of oxidative stress in the toxicity of glyphosate and its co-formulants regarding immuno-hematological cell functions.

The complement system is an important component of innate immunity in teleost fish and can be affected by various abiotic stresses [6], including chemical exposure [72]. Different effects of GBHs on fish complement have been reported, depending in particular on the exposure dose. Kreutz et al. [41] found no impact of sub-chronic exposure to a concentration of 0.730 mg L^{-1} of a GBH on complement hemolytic activity in the serum of goldfish (*Carassius auratus*), while Ma et al. [49] reported that chronic exposure to doses of 52.08 and 104.15 mg L^{-1} of a GBH reduced both the expression and the content of complement (C3) in the kidney of common carp (*Cyprinus carpio*). At our level of contamination, no impact in hemolytic activity of alternative complement pathway was detected.

Cytokines are secreted proteins that are important for the development and regulation of fish immune responses. Among them, $TNF-\alpha$ and $IL1 - \beta$ act in inflammation and regulation of immune cells [73]. The expression of both these cytokines showed a decreasing (non-significant) trend in fish exposed to GBHs and this trend was also observed to a lesser extent for fish exposed to the AS. As some of these cytokines, like $IL1 - \beta$, are produced by macrophages [34, 73], there can be a direct correlation between their level of expression and variations in the population of these immune cells. Disruption of the specific immune

response set up to fight against an infectious agent could be detrimental for fish health and complementary studies must be done to confirm these tendencies.

Outside of the spawning period, where mortality events occurred in control and contaminated groups, no abnormal behavior or clinical signs were observed. Reproduction periods mobilize a large amount of energy and are frequently associated with greater fragility, making fish more sensitive to external aggression (e.g. pathogens, sampling events; [11]). No significant differences between conditions occurred in ponderal growth during the period of gonad maturation and spawning. Fish exposed to Roundup showed slightly lower weights, which could suggest an increase in energy demand due to detoxification and repair processes, leaving less energy available for growth and reproduction [76]. The lower growth observed for the Roundup condition could be the result of an investment of the extra-energy produced in reproduction rather than in growth. This phenomenon has already been observed in RT, and different trade-offs can be seen depending on stress events [11]. Fecundity and fertility were studied using different markers on mature adult RT, and no impact of chemical exposure was shown. If a specific mechanism of reproductive toxicity occurred, such as hormone disruptions, it did not affect reproductive parameters measured here. Uren Webster et al. [84] reported effects on steroidogenic biosynthesis pathways in zebrafish (*Danio rerio*), but no disruption of spermatogenesis and fertilization after simple or complex exposure at 10 mg L^{-1} of glyphosate. In their study, egg production was impacted with pure glyphosate but not with GBH, which could act to maintain or restore the estrogen signaling pathway. Even though, in the present study, the very low environmentally relevant exposure of adult RT to glyphosate and GBHs might not affect reproductive parameters, it would be interesting to investigate if an impact may still occur on F1 generation due to exposure of gametes during gametogenesis.

5. Conclusions

This study investigated the impact of long-term chronic exposure to a low-dose of glyphosate in adult rainbow trout through the observation of a wide variety of parameters e.g. reproduction, oxidative response, energetic metabolism and immune system, including a comparison between the AS and two GBHs. The results obtained revealed no major changes for the majority of markers analyzed except, occasionally, some elements of the immune response such as the proportion of macrophages in the blood or phagocytic capacities. Interestingly, these significant modifications were observed only for the formulated products, suggesting a potential role of the co-formulants in the induced effect. The environmentally relevant exposure concentration of glyphosate of less than $1 \text{ } \mu\text{g L}^{-1}$ we used is probably too low to induce detectable changes over the 10-month period in adult fish. It is important to note that although our experimental conditions were optimal for the physiology of RT, the toxicity of glyphosate and GBH co-formulants in the real aquatic environment could be enhanced by biotic and abiotic stressors, and interaction with other chemicals - i.e. a cocktail effect. Complementary studies are ongoing on F1 and F2 generation derived from the RT used in this study to explore the potential effects of a direct interaction of chemicals with germinal cells but also of a transgenerational exposure.

Competing interests

The authors declare that they have no conflict of interest.

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Appendix A. Growth parameters

Table A.7: Mean weights of mature RTs before and after spawning period

Condition	Weights		
	before spawning	after spawning	ratios before/after
C	1208 ± 117	1387 ± 213	1.14 ± 0.13
G	1131 ± 193	1249 ± 222	1.11 ± 0.11
R	1252 ± 337	1230 ± 221	1.03 ± 0.25
V	1119 ± 245	1269 ± 189	1.15 ± 0.11

Weights values expressed as : mean (g) ± standard-deviation (g)

Weight before spawning have been measured in September 2018, weight after spawning have been measured in November 2018.

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