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Immunological and metabolic effects of acute sublethal exposure to glyphosate or glyphosate-based herbicides on juvenile rainbow trout, Oncorhynchus mykiss

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Abstract

Glyphosate is a commonly used agrochemical active substance co-formulated in glyphosatebased herbicides (GBHs) whose environmental safety is still a subject of debate in the European Union. We evaluated the effects of acute sublethal exposure to glyphosate on rainbow trout by measuring changes in their metabolic and hemato-immunologic functions and their ability to survive a viral challenge. Juvenile fish were exposed for 96 h to 500 μ g L⁻¹ of glyphosate through the active substance alone or two GHBs, Roundup Innovert[®] and Viaglif Jardin[®], and fish were then infected with the infectious hematopoietic necrosis virus. Red and white blood cell counts (RBCC and WBCC), as well as several enzymatic activities (citrate synthase, CS; cytochrome-c oxidase, CCO; lactate dehydrogenase, LDH; glucose-6phosphate dehydrogenase, G6PDH; acetylcholinesterase, AChE), were measured 96 hours after chemical contamination (S1), and 96 hours post-viral infection (S2). Mortality rates were monitored, and virus titers at the mortality peaks and seropositivity of the survivors were analyzed at 60 days post-viral infection (S3). Cumulative mortalities, viral titers, and seropositivity induced by virus infection were similar among conditions. Hematological analysis revealed significant increases of 30% for RBCC for Roundup at S1, and of 22%for WBCC at S2. No changes were observed in metabolic enzyme activities at S1. At S2, CCO and G6PDH activities were significantly higher than controls in all the chemically contaminated groups (+61 to 62% and +65 to 138%, respectively). LDH and AChE activities were increased for the Viaglif (p = 0.07; +55%) and for glyphosate and Roundup conditions (p < 0.05, + 62 to 79%), respectively. Rainbow trout acutely exposed to glyphosate or GBHs presented no major physiological changes. Viral infection revealed disruptions, potentially modulated by co-formulants, of hematological and metabolic parameters, showing that it is essential to consider the stressful natural environment of fish in the chemical assessment. *Keywords:* Glyphosate, Rainbow trout, Energy metabolism, Immune function, Viral challenge

1. Introduction

Freshwater ecosystems play a critical role in maintaining both human and environmental health. They have important ecological functions, maintain high biodiversity and provide 5 goods and services to human societies as a source of both food and water [4]. Water quality 6 is impacted by chemical substances originating from human activities such as industry or agriculture [43]. Among them, agrochemicals, such as the widely used herbicide glyphosate, 8 are a particular threat to their integrity and this trend could be increased in a context 9 of global climate change, where different biotic and abiotic stressors might interact [74]. 10 Ecotoxicology aims to evaluate the impact of these chemical contaminants on ecosystems 11 and to define regulatory limits that maintain the use of these compounds under sustainable 12 levels [5]. Glyphosate is the active substance (AS) of glyphosate-based herbicides (GBHs), 13 which are the most commonly used pesticides worldwide. Concerns have been raised about 14 its environmental safety [69, 68] and re-registration of this compound and its associated 15 commercial products is still the subject of debate at the level of the European Union [64]. 16 While glyphosate shows low potential for bioaccumulation in animal tissues [22], low levels 17 of this chemical are ubiquitously encountered in the environment and aquatic organisms are 18 therefore continuously exposed. In France, from 2007 to 2017, glyphosate detection in surface 19 water increased from 22.2 to 49.7% of sampling points analyzed, and the mean maximum 20 annual concentrations quantified during this interval among all the sampling points ranged 21

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from 2.4 to $70.2 \,\mu g \, L^{-1}$. However, the same report revealed that only one analysis among all sampling points in 10 years was higher than the reported value of Predicted No Effect Concentration (PNEC of $60 \,\mu g \, L^{-1}$, determined using both acute and chronic toxicity values) [3]. These results for measured glyphosate concentrations are consistent with the Predicted Environmental Concentration (PEC) of $104.8 \,\mu g \, L^{-1}$ reported by the European Food Safety Authority (EFSA) when 'realistic worst-case' exposure scenarios are considered [22].

Toxic effects of acute glyphosate exposure have already been observed at several levels of 7 biological organization. Acute exposure is defined as contact between the organisms (fish) 8 used as indicators and the chemical to be tested, less than or equal to 96 hours [52]. This 9 approach is rapid and convenient to define the dose inducing 50% mortality (LC50) in a 10 specific species [51]. LC50_{96 h} values comprised between 22 and $> 1000 \text{ mg L}^{-1}$ for the AS 11 and comprised between 4.2 and 52 mg L^{-1} for a GBH were determined for several fish species, 12 including several Salmonidae [26]. In rainbow trout (RT), one of the most important farmed 13 fish species in Europe and a relevant model in ecotoxicology, EFSA [22] recently reported 14 an LC50 value of $38 \,\mathrm{mg}\,\mathrm{L}^{-1}$. This LC50 value is at least about 362 times higher than the 15 PEC calculated by EFSA, and acute exposure to glyphosate is therefore unlikely to have 16 a significant environmental impact. For the present study, we chose a concentration of 17 AS 76 times lower than the LC50 reported by European Food Safety Authority [22] that 18 would make it possible to detect changes specific to the glyphosate mode of action (MoA), 19 rather than non-specific disruptions induced by high toxic stress. However, several studies 20 have suggested that sublethal doses of glyphosate, administered as the AS or GBH, could 21 induce biological or physiological alterations which might affect wild fish living in a complex 22 and stressful environment [38, 41, 46, 23, 28, 53], impacting their thermal tolerance [76] or 23 pathogen susceptibility [39]. Immune toxicity of glyphosate has been demonstrated in certain 24 in vitro assays [20, 70]. Moreover, the fish immune system seems to be particularly influenced 25 by exposure to pure glyphosate AS [42, 71, 45] or to GBHs [37, 23, 38, 47]. However, 26 studies linking disruptions of hemato-immunological parameters to pathogen susceptibility 27 of exposed fish are still lacking. Energy metabolism is another critical function involved 28 in the adaptation of an organism facing multiple stressors, e.g. by compensating for the 29

increase in energy demand due to higher maintenance costs [63]. Acute exposure of fish to GBHs shows direct or indirect effects of glyphosate and its co-formulants on energy metabolism [21, 28, 29, 27, 67, 40] that could reveal a lower ability to adapt to and therefore face a pathogenic infection. The MoA of glyphosate and GBHs in non-target species is not yet well understood [2, 49].

In this study, we evaluated the effects of acute sublethal exposure to glyphosate administered alone or through two GHBs on rainbow trout. Several physiological functions were investigated at different levels of biological organization. Moreover, a viral challenge was used to investigate the ability of glyphosate-contaminated trout to implement appropriate metabolic and immune responses to survive this infection.

2. Materials and methods

2.1. Fish maintenance

A total of 420 specific pathogen-free (SPF) juvenile rainbow trout (RT) four months old 13 (Mean \pm standard deviation = 2.8 g \pm 0.8 g) from the protected and monitored fish facilities 14 of the ANSES Plouzané Laboratory site (France) were used. The fish growth period was 15 conducted in 12 tanks (30 L) positioned in a confined room, supplied with an open circuit 16 with filtered river water and equipped with adapted aeration to maintain oxygen levels 17 between 6 and 8 mg L^{-1} . Every morning, after fish care and tank maintenance, trout were 18 fed with trout-specific food (Neo supra AL4, Le Gouessant^{\mathbb{R}}), at 1.5% of biomass. The 19 same photoperiod (12 h of daylight) was maintained throughout the experiment. Water 20 temperature during the chemical exposure was maintained at $15 \,^{\circ}\text{C}\pm2 \,^{\circ}\text{C}$, while during the 21 viral challenge water temperature was set to $11 \,^{\circ}C \pm 2 \,^{\circ}C$ to ensure a range within the thermal 22 optimum of the virus. 23

2.2. Contaminants

Glyphosate (G; Sigma-Aldrich, ref. 45521, CAS Number 1071-83-6), Roundup Innovert[®] (R; Agrilisa) and Viaglif Jardin[®] (V; Agrilisa) were tested. 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 27

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concentrations of the co-formulants of the two commercial products (R and V) are unknown, ¹ but they were formulated for two different uses. R was formulated for professional use, while ² V was formulated for home gardens. Concentrated solutions for each product (200 mg L^{-1}) ³ were prepared and stored at room temperature and in dark conditions to perform the whole ⁴ 96 h experiment. ⁵

2.3. Virus production and titration

The virus used for viral challenge was the N61 strain (genotype E) of the infectious 7 hematopoietic necrosis virus, i.e. *IHNv*, isolated from diseased rainbow trout fry displaying 8 typical signs of the disease. A 100 mL stock of virus was produced at 14 °C on an *Epithe*-9 *lioma Papulosum Cyprini* (EPC) cell line [24] in Tris-buffered Stoker's medium (pH 7.6), 10 supplemented with 10% fetal bovine serum (FBS), as described by Dupuy et al. [17]. Once 11 the cytopathic effect was complete, cell culture supernatant was centrifuged for 15 min at 12 $2,000 \times q$ and stored at -80 °C. The infectious titer of the viral production, determined 13 using the 50% tissue culture infective dose (TCID₅₀) end-point method in 96-microplate 14 wells [34], was $4 \times 10^7 \,\mathrm{TCID}_{50} \,\mathrm{mL}^{-1}$. 15

2.4. Experimental design

2.4.1. Ethics statement

All animal studies were carried out in the approved infrastructure of the French Agency 18 for Food, Environmental and Occupational Health & Safety (ANSES) at Plouzané (France; 19 approval number D29-212-03), in strict accordance with the European guidelines and rec-20 ommendations on animal experimentation and welfare (European Union Directive 2010/63). 21 All animal experimental procedures were analyzed by the ethics committee on animal ex-22 perimentation ANSES/ENVA/UPC No. 16 and were authorized by the French Ministry of 23 Research, under the number APAFIS #2018020115216522. Euthanasia involved the addition 24 of a lethal dose of 100 ppm of Eugenol into the tank water. 25

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2.4.2. Chemical exposure system

Four conditions of chemical contamination were tested in triplicate (i.e. a total of 12 tanks of 30 L; Figure 1b). Each replicate included 150 rainbow trout as the chemically unexposed control (C), the group exposed to glyphosate AS (G), or exposed to GBHs, Roundup Innovert (R) and Viaglif Jardin (V). Chemical treatments were carried out for 96 hours in semistatic river water, injecting a volume of 75 mL of each concentrated solution to obtain a final theoretical exposure concentration of 500 μ g L⁻¹ of glyphosate (1b). Every 24 hours, contaminated water was entirely renewed and chemical contamination was reproduced.

2.4.3. Viral challenge

After 96 h of chemical exposure, 99 fish for each chemical treatment (i.e. 33 fish per replicate of a condition) were randomly distributed in four 10 L tanks with constant water renewal (i.e. three tanks were infected with the *IHNv* and one was used as the uninfected viral control; Figure 1b). Infection was done by challenging fish for 3 hours in a reduced volume of 1 L highly oxygenated water with an infectious dose of $10^4 \,\mathrm{TCID}_{50} \,\mathrm{mL}^{-1}$. Fish in viral uninfected control tanks were challenged in the same conditions with non-infected EPC cell supernatant.

During the viral challenge, general behavior, possible specific clinical signs of rhabdovirosis, and mortality were recorded twice a day. Dead individuals were weighed and stored 18 at -20 °C for viral examination. 19

2.5. Samples and sampling date

After the first exposure day, 150 mL of water were sampled in one replicated tank, before ²¹ and after water renewal to quantify the glyphosate concentration in water and to ensure ²² that chemical exposure was stable over 96 h. Samples of water were stored at -20 °C before ²³ the chemical analysis. ²⁴

Three invasive sampling dates were performed in the four conditions, i.e. 96 h after the ²⁵ chemical exposure (S1; 15 fish per replicate of chemical treatment; n = 45), 96 h post-²⁶ infection (S2; 15 fish per replicate of chemical treatment infected by *IHNv*; n = 45) and ²⁷

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Figure 1: Timeline (a) and experimental design (b). Fish were exposed for 96 hours to clear water (in blue) or to 500 µg L⁻¹ of glyphosate through the AS (in yellow) or the two GBHs (R in orange and V in purple) (3 tanks of 30L per condition, n = 150 by tanks). After acute exposure, 45 fish (15 fish per tank and condition) were sampled for analyses (S1). Residual fish were then distributed to 4 tanks of 10 L per condition (n = 99 by tanks) for the viral challenge. Three tanks were infected with *IHNv* (virus symbol) and the last one was used as a non-infected control. 15 fish per tank (45/condition) were sampled at 96 hours post-infection (S2). Survivors were sampled at 60 dpi (S3) and then euthanized.

60 days post-infection (dpi; S3; 5 survivors by replicate of chemical treatment; n = 15). ¹ For each fish, a blood sample was taken by withdrawing blood from the caudal vein with a ² lithium heparin hematocrit tube (Greiner ref. KG454244). At S1 and S2, 5 µL of whole blood ³ was used for hematological analyses. At S3, 50 µL of whole blood was sampled and then ⁴ centrifuged (1, 200 × g, 10 min, 4 °C) and only plasma was stored at -80 °C for serological ⁵ analysis. At S1 and S2, whole fish bodies were flash-frozen in liquid nitrogen and stored at ⁶ -80 °C for future analyses. ⁷

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2.6. Chemical and biological parameters

2.6.1. Chemical parameters

Quantification of glyphosate using direct competitive ELISA assay (Novakits, ref. 1500086) 10 was done before and after the water renewal, following the supplier's instructions. Briefly, 11 the river water was first filtered at $0.2 \,\mu\text{m}$ (Clearline ref. 146560), 250 μL of the filtrate 12 was derivatized with $100\,\mu\text{L}$ of the derivation solution provided in the kit. Then, $50\,\mu\text{L}$ 13 was transferred to wells in a microplate coated with goat anti-rabbit antibodies and $50\,\mu\text{L}$ 14 of the reagent containing rabbit anti-glyphosate antibody was added. A competitive reac-15 tion for binding sites (between glyphosate and glyphosate enzyme conjugate) was started 16 by the addition of $50\,\mu\text{L}$ of enzyme conjugate solution for 1 h. After a washing step using 17 $3 \times 250 \,\mu\text{L}$, $150 \,\mu\text{L}$ of the substrate solution was added into the wells and the colored reac-18 tion was stopped after 25 min by adding 100 µL of the stop solution. Plates were read at 19 450 nm on a TECAN Spark 10M microplate spectrophotometer. The development of color 20 is inversely proportional to the concentration of glyphosate in the sample. Standard curves 21 were generated using the four-parameter log-logistic function, LL.4, of the R package "drc" 22 [61]. Quantification was done by reporting the OD value obtained for the sample to the 23 quantification standard curve obtained for each assay. 24

Quantification of glyphosate and aminomethylphosphonic acid (AMPA) by HPLC/fluorometacic methods (Method ref. ANA-I10.MOA.69.B) was done by an external provider (Labocea, ²⁶ France) only after the first water renewal and recontamination, in one tank per condition. ²⁷

2.6.2. Viral examination in fish

The presence and concentration of virus were checked individually from 3 dead fish collected at the peak of mortality, by replicate of chemical conditions. Extracted and pooled organs (kidneys, spleen, heart, and brain) were crushed using a mortar and pestle, diluted to 10^{-1} with Stoker's medium, and centrifuged for 15 min at $1,500 \times g$ at 4 °C. The supernatant swas then diluted to 10^{-11} , and the virus concentration was determined for each fish, as described in section 2.3.

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2.6.3. Immune parameters

At S1 and S2, counting of red and white blood cells was performed on a Thoma cell $_{9}$ hemocytometer using whole blood diluted to 1/200 in Giemsa solution [35]. The alternative $_{10}$ pathway of plasma complement activity was measured according to Danion et al. [13]. Results were expressed using the formula described by Costabile [12]. At S3, the detection and $_{12}$ semi-quantification of anti-*IHNv* antibodies in the plasma of surviving fish were performed $_{13}$ using a modification of the procedure of Jorgensen et al. [33] described by Dupuy et al. [17]. $_{14}$

2.6.4. Bio-marker, oxidative stress, and metabolic parameters

Each assay measurement was performed in triplicate. Whole fish were dry-homogenized ¹⁶ using the tissue homogenizer Precellys 24 (Bertin Technologies, France) and homogenates ¹⁷ were then diluted in phosphate buffer (0.1 M, pH 7.8). Colorimetric analysis was carried ¹⁸ out on a TECAN Spark 10M microplate spectrophotometer. Choline esterases (ChE), thio-¹⁹ barbituric acid reactive substances (TBARS), citrate synthase (CS), cytochrome-c oxidase ²⁰ (CCO), lactate dehydrogenase (LDH), and glucose-6-phosphate dehydrogenase (G6PDH) ²¹ were measured following procedures described by Pédron et al. [55] and Gauthier et al. [25]. ²²

Enzymatic activities were calculated using the slope of the optical curve density = 23 f(time) and the Beer-Lambert law ($A = \epsilon lc$) for ChE, CS, CCO, LDH and G6PDH (with 24 molar extinction coefficient, ϵ , of 13.6, 13.6, 21.84, 6.22 and 6.22 L mol⁻¹ cm⁻¹ respectively); 25 and with a calibration curve for TBARS. Calibration curves were generated using 1,1,3,3- 26 tetramethoxypropane (Sigma-Aldrich ref. T9889) for the TBARS assay. Protein concentra- 27 tions were measured in all organs using the Pierce BCA protein assay kit (ThermoFisher 28 Scientific), and results are expressed in specific activities $(IU mg^{-1})$.

2.7. Data processing and statistical analyses

Data processing and statistical analyses were conducted with R software [57]. Figures 3 were produced using the ggplot2 package [73]. All the data were tested for normality and 4 homoscedasticity. In the case of normal and homoscedastic data, one-way Anova tests were 5 used to compare means, followed by a post-hoc test of Dunnett [16]. In the case of normal 6 and heteroscedastic data, modified one-way Anova tests were used to compare means [72], 7 followed by a post-hoc test of Tamhane-Dunnett [50]. In the case of non-normal data, a 8 Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn [15]. 9 Differences between proportions of seropositive individuals between chemical treatments 10 were compared using a Chi-squared test. A p-value of 0.05 was used as the threshold for 11 statistical significance. A test of correlations between variables was carried out using the 12 correlation of Pearson. Mortality rates for the different chemical treatments were compared 13 using the "survival" package [65] by following the procedure described by Doumayrou et al. 14 [14]. Restricted mean survival time, an integrated parameter representing both the dynamics 15 and the intensity of mortality [65], was calculated using the "survival" package. 16

3. Results

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3.1. Determination of glyphosate concentrations during acute contamination

Glyphosate and AMPA were not detected in control tanks, and AMPA was not detected 19 in any of the contaminated samples analyzed. Concentrations of glyphosate were between 20 427.82 and $497.77 \,\mu g \, L^{-1}$ for all conditions in the contaminated tanks at the end of the first 24 21 hours of contamination, but also after water renewal and re-contamination using an ELISA 22 assay, except for the Roundup condition where a higher concentration (728.35 μ g L⁻¹) was 23 detected after re-contamination. Quantifications done by HPLC yielded similar concentra-24 tions, between 428.80 and 477.00 μ g L⁻¹. Regardless of the method considered and excluding 25 the value of $728.35 \,\mu g \, L^{-1}$ obtained using ELISA for the Roundup condition, glyphosate con-26 centrations obtained in contaminated tanks did not show a difference greater than 15% from the expected values (i.e. $500 \ \mu g \ L^{-1}$). (Table 1).

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Table 1: Concentrations of glyphosate $(\mu g L^{-1})$ measured in water tanks using ELISA (n = 2) and HPLC (n = 1) methods. ELISA values are expressed as mean \pm standard error. nd = non-detected; nm = non-measured.

Sampling	Condition	Glyphosate concentration $(\mu g L^{-1})$		
8		ELISA	HPLC	
	\mathbf{C}	nd	nd	
After 24h	G	460.48 ± 77.92	nm	
of exposure	\mathbf{R}	497.77 ± 11.55	nm	
	\mathbf{V}	427.82 ± 4.45	nm	
	C	464 40 1 59 15	477	
After water renewal	G	404.48 ± 53.15	477	
and recontamination	\mathbf{R}	728.35 ± 12.15	466	
	\mathbf{V}	442.70 ± 26.30	428.8	

3.2. Survival rates during chemical exposure and viral challenge

No mortality was recorded during the 96 h exposure period to glyphosate and GBHs. 4 During the viral challenge, while no mortality occurred in the non-infected control fish, 5 mortality in infected tanks began overall at 4 or 5 dpi. At 60 dpi, cumulative mortality 6 (mean \pm se, n=3) reached $61.8 \pm 0.9\%$ for the control, $60.4 \pm 6.2\%$ for glyphosate, $64.9 \pm$ 7 2.2% for Roundup, and $67.8 \pm 4.5\%$ for Viaglif (Figure 2), without significant differences in 8 mortality between groups. Slightly faster and higher mortality was observed in fish exposed 9 to Roundup and Viaglif, with shorter restricted mean survival times (RMST) of (mean \pm 10 se, n = 3) 33 ± 1.0 and 31 ± 2.6 dpi respectively, compared to 35 ± 0.7 and 36 ± 3.0 dpi for 11 the control and glyphosate conditions. 12

Maximum daily mortality (i.e. peak mortality) was observed at 6 dpi for the control and Viaglif, and at 8 dpi for glyphosate and Roundup. A second mortality peak was observed 2 between 12 and 13 dpi particularly for Roundup and to a lesser extent for the control and 3 glyphosate. Pools of infected fish that had died at the first mortality peak were all positive to *IHNv* for the control and Roundup conditions, 2/3 and 1/3 of fish were positive for the glyphosate and Viaglif conditions, respectively (Table 2). The viral titers were not drastically different among conditions of chemical contamination but presented a high variance among analyzed pools. 8



Figure 2: Kinetics of cumulative mortality in fish exposed to chemical contamination and infected with IHNv. Data are expressed as a function of time in days post-infection (dpi) for the glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue) groups. Error bars represent standard errors, vertical bars the restricted mean survival time (RMST).

3.3. Immune parameters

After 96 h of acute exposure to glyphosate (S1), RBCCs were increased with the AS and 10 the two GHBs compared to the control, particularly for fish exposed to Roundup with a 11 significant rise of 35% (p < 0.05; Figure 3a). WBCCs did not vary significantly, regardless 12 of the chemical contamination (Figure 3b). 13

After 96 h of viral infection (S2), no significant differences were measured in the RBCCs 14 between controls and exposed fish. WBCCs in fish exposed to Viaglif were significantly 15

Table 2: Proportion of *IHNv*-positive pools of fish exposed to glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue), and mean viral titers measured at the mortality peak (mean \pm standard-error, n = 3).

Parameter	Condition				
	С	G	R	V	
Proportion of <i>IHNv</i> -positive fish	3/3	2/3	3/3	1/3	
IHNv titer	4.86×10^4 (4.37×10^4)	7.12×10^4 (6.48 × 10 ⁴)	4.58×10^5 (4.51×10^5)	6.32×10^5	

higher than controls (22%; p < 0.05).

At 60 dpi (S3), similar proportions from 67 to 80% of the survivor fish analyzed by condition were seropositive for *IHNv* (Table 2). The control and glyphosate conditions were characterized by percentages of highly *IHNv* seropositive fish (i.e. presenting an antibody titer greater than 640), which was higher than but not significantly different from the GBHs, with 70 and 64% versus 50 to 55%, respectively (Table 3). Antibody titers of highly *IHNv* seropositive fish presented a high variance for each condition, without any differences between their means (Table 3).

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3.4. Oxidative stress and metabolic parameters in whole fish

No significant differences were observed in CS, CCO, LDH, and G6PDH activities after 10 the 96 h acute exposure period to glyphosate and GBHs. After the viral infection, CCO 11 activities were significantly higher in all the chemically contaminated groups (p < 0.05), 12 with differences of 61, 62, and 61% with the control group for glyphosate, Roundup, and 13 Viaglif, respectively (4). LDH activities showed a tendency to increase in fish exposed to 14 contaminants compared to the control, but this increase was only significant for Viaglif (+ 15 55%; p < 0.05; 4). Interestingly, the Viaglif tank associated with lower LDH activity also 16 presented low mortality, and when all replicates were put together (i.e. infected fish in all 17





Figure 3: Barplot representing mean red blood cell count (RBCC) and mean white blood cell count (WBCC) in fish exposed to glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue) at S1 (post contamination) and S2 (post infection). Standard errors are represented at the top of each bar ($20 \le n \le 33$). Statistically significant differences from the control mean are indicated with "*" (p < 0.05); comparisons were made between groups at the respective sampling times.

Table 3: Proportions of seropositive and highly seropositive fish and mean anti-*IHNv* antibody titers (mean \pm standard-error, n = 15) at 60 dpi (S3) in fish previously exposed to glyphosate (G), Roundup (R), Viaglif (V) and control (C). The proportion of highly seropositive fish corresponds to the ratio between the number of fish with a titer \geq 640 and the total number of seropositive fish. Mean antibody titers were calculated for highly seropositive fish only.

Paramotor	Condition			
Parameter Proportion of seropositive fish Proportion of highly seropositive fish (% Anti- <i>IHNv</i> antibody titer	С	G	R	V
Proportion of seropositive fish	10/15	11/15	11/15	12/15
Proportion of highly seropositive fish (%)	70	64	55	50
Anti- <i>IHNv</i> antibody titer	1189	914	2240	1280
	(259)	(129)	(656)	(405)

chemical exposure conditions taken together), a moderate correlation was observed between 1 LDH activity and mortality (Pearson's correlation = 0.55, p = 0.07). The ratio between 2 LDH and CS activities presented high variability, regardless of the chemical condition and the 3 sampling time considered. No significant differences were observed between the treatments before and after the viral challenge. Ratios were comprised between 471 and 528% after the 5 chemical contamination, and between 611 and 777% 96 h after the viral infection. The ratio 6 between CS and CCO activities was significantly higher in fish contaminated by Roundup 7 after 96 h of chemical exposure (p < 0.05) compared to the other conditions (128% for the 8 control compared to 150% for the Roundup condition, a difference of 17%). After the viral 9 infection, this parameter presented differences of 26, 27, and 32% for glyphosate, Roundup 10 and Viaglif compared to the control (p < 0.05), respectively. Ratios were comprised between 11 121 and 136% for chemically contaminated fish and equal to 190% for the controls. G6PDH 12 activities in infected fish exposed to glyphosate and GBHs were increased compared to 13 the controls. This tendency was particularly marked in GBH conditions, with observed 14 differences of 126 and 138% for Roundup and Viaglif (p < 0.05), respectively (4). TBARS 15

concentration was not significantly different among conditions either after the acute chemical ¹ exposure or the viral infection. ²

No significant differences were observed in AChE activities between fish after the 96 h $_{3}$ of acute chemical contamination. The enzymatic activity was increased in glyphosate and $_{4}$ Roundup conditions after the viral challenge, by 62 and 79%, respectively, compared to the $_{5}$ control (p < 0.05) (Figure 4).



Figure 4: Mean specific enzymatic activities of AChE in fish exposed to glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue) at S1 (post contamination) and S2 (post infection). Enzymatic activity is expressed in IU mg⁻¹ of protein. Standard errors are represented at the top of each bar (n = 15 for sample post contamination and $8 \le n \le 15$ post infection). Significant differences from control means are indicated with "*" (p < 0.05); comparisons were made between groups at the respective sampling times.

Table 4: Mean specific activities and TBARS levels measured in fish exposed to glyphosate (G), Roundup (R), Viaglif (V) and control (C) at S1 (post contamination) and S2 (post infection). Standard errors are represented in parentheses under each respective mean ($9 \le n \le 15$ at S1 and $7 \le n \le 15$ at S2). Specific activities are expressed in IU mg⁻¹ of protein and TBARS levels in nmol mg⁻¹ of protein. The numbers in bold with an asterisk are significantly different (p < 0.05) from the values obtained for the control condition at the same sampling time

Sampling	Condition	Enzymatic parameters				
	Condition	CS	CCO	LDH	G6PDH	TBARS
	С	0.39	0.31	2	0.0121	0.43
		(0.024)	(0.024)	(0.14)	(0.0013)	(0.097)
	G	0.42	0.31	1.95	0.0156	1.72
		(0.034)	(0.0167)	(0.2)	(0.0017)	(0.65)
post conta.	\mathbf{R}	0.41	0.27	2.25	0.016	0.88
		(0.021)	(0.011)	(0.43)	(0.0016)	(0.36)
	\mathbf{V}	0.33	0.28	1.57	0.014	0.71
		(0.021)	(0.015)	(0.083)	(0.00099)	(0.31)
	С	0.26	0.15	1.5	0.0046	0.86
		(0.021)	(0.014)	(0.1)	(0.0016)	(0.31)
	G	0.33	0.24^{*}	1.8	0.0076	0.59
		(0.023)	(0.0065)	(0.17)	(0.002)	(0.16)
post inf.	R	0.32	0.24^{*}	2.03	0.01*	0.84
		(0.023)	(0.017)	(0.2)	(0.0013)	(0.45)
	\mathbf{V}	0.29	0.24^{*}	2.33*	0.011*	0.38
		(0.016)	(0.013)	(0.204)	(0.00132)	(0.043)

4. Discussion

This study was designed to evaluate the impact of acute exposure to a sublethal concentration of glyphosate on RT, integrating a comparison of the effect of the AS alone or associated with co-formulants in two GBHs.

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Glyphosate concentrations during chemical exposure were close to $500 \,\mu g \, L^{-1}$, regard-5 less of the contaminants used, except in the Roundup tank where the concentration was 6 46% higher than the expected value. This might be explained by early sampling of wa-7 ter, before complete homogenization of the concentrated solution in the tank volume. The 8 ELISA method, which is practical and not expensive, appears to yield results similar to the 9 HPLC method in most cases, except one probable overestimation potentially due to inter-10 ference between water components and chemicals used in the assay. In this project, water 11 used to supply the fish tanks did not contain glyphosate or AMPA at detectable levels and 12 AMPA was not detected in contaminated tanks. The dose selected for acute testing, i.e. 13 $500 \,\mu g \, L^{-1}$, is unlikely to be encountered in Europe since the estimated global maximum 14 PEC for surface water reported by EFSA is $104.81 \,\mu g \, L^{-1}$ [22]. However, this concentration 15 might be environmentally relevant in countries where the cultivation of genetically mod-16 ified glyphosate-resistant crops is authorized [7]. Also, the scenario of acute exposure to 17 glyphosate or GBHs is more likely when glyphosate is directly sprayed into the aquatic 18 environment (e.g. algae control [30]; [26]). 19

At this concentration, the AS associated or not with co-formulants did not induce mortality after 96 h of acute exposure. The exposure dose is relatively low compared to the resistance of rainbow trout to acute contamination by glyphosate or GBHs [22, 26]. Effects observed in this configuration probably result from specific effects, directly related to the MoA of glyphosate and its co-formulants, rather than general cell or organism dysfunction typically induced by an excessively high test dose. Thus, our experimental design was able to reveal the sublethal effects of glyphosate exposure in rainbow trout. 20

The innate and specific immune systems play a fundamental role in intensive aquaculture ²⁷ and the natural environment where fish are threatened by a large diversity of pathogens [11]. ²⁸ Several studies have reported the effects of pollutants on fish immune functions (reviewed by Rehberger et al. [58]). Nevertheless, except for findings at low levels of biological organi-2 zation, such as the molecular [42, 71] or cellular levels [45, 37, 23, 38, 47], only few data are 3 available on the effects of glyphosate on overall immune system functioning. Nonetheless, 4 studies using pathogens with direct fish infections after a period of chemical exposure are 5 particularly useful to detect the immunotoxic effects of chemical contaminants [58]. To our 6 knowledge, no studies using viral challenge after exposure to glyphosate or GBHs are avail-7 able in the literature. Kreutz et al. [39] have already shown that acute exposure of silver 8 catfish fingerlings, *Rhamdia quelen*, to $730 \,\mu g \, L^{-1}$ of a GBH induced changes in immune cells 9 and consequently higher susceptibility to the bacterium Aeromonas hydrophila. 10

In our study, detection of the virus in fish that died during the challenge confirms the 11 efficiency of the infection process, even though differences in the number of IHNv positive 12 individuals were observed depending on the conditions. These differences are probably asso-13 ciated with infectivity loss of some samples induced by poor conservation of fish upstream of 14 the cell culture. This hypothesis is supported by the high seropositivity rates for *IHNv* mea-15 sured in the survivors, evidence of efficient entry of the virus. The frequency of seropositivity 16 observed in trout was not impacted by chemical exposure. Despite this, the proportion of 17 highly seropositive fish tended to be lower in conditions with GBH exposure (no statistical 18 significance). Consequently, further studies could provide evidence that GBHs reduce the 19 specific immune response of fish. On the other hand, the dynamics and intensity of mortal-20 ity were very similar between control fish and those contaminated with the AS. However, 21 slight differences in the dynamics of mortalities (i.e. faster mortalities) and survival rates 22 (i.e. higher mortalities) were observed for fish exposed to GBHs, particularly to Viaglif, but 23 without statistical significance. These results are, therefore, different from those reported 24 by Kelly et al. [36] and Kreutz et al. [39] who found increased pathogen susceptibility of fish 25 due to co-formulated glyphosate exposure at concentrations of AS close to ours. Also, it is 26 important to note that in our experiment, fish were not chemically exposed during the viral 27 challenge (only before), and survival following the viral infection might be more strongly 28 affected when fish are both exposed to the virus and chemical contaminants. The slight 29

tendencies we observed will have to be confirmed to determine whether, when using higher concentrations (particularly with exposure during the viral challenge) or longer exposure times and testing other viral species, some co-formulants may influence the survival of fish facing viral infection.

Another study, where fish were not exposed to a pathogen, has shown that 7 days of 5 exposure of common carp, Cyprinus carpio, to a GBH, at concentrations corresponding to 6 0.5 and 5.0 mg/L of glyphosate, induced immunosuppression balanced by a compensatory 7 response of the hematopoietic system [37]. Hematology and more specifically, cellular numer-8 ation, are often used to evaluate effects induced by pollutants on fish [8]. In our experiment, 9 the 96 h period of chemical contamination induced an increase in RBCCs in fish exposed to 10 Roundup. This result was previously reported for the neotropical fish, *Prochilodus lineatus*, 11 exposed to 5 mg L^{-1} but not to 1 mg L^{-1} [46]. This increase, not triggered by pure glyphosate 12 or Viaglif, could be a protective response to the toxic effect of the specific Roundup formu-13 lation or a result of detoxification of the chemical compounds included in this formulation. 14 Also, alone, the chemical contamination had no impact on the leukocyte counts, but a signif-15 icant increase was observed for the Viaglif condition after the viral infection. Interestingly, 16 when the WBCCs were compared overall before and after the viral infection, leucopenia was 17 observed after the infection with *IHNv*. This leucopenia is not commonly observed under a 18 viral infection challenge in rainbow trout [1]. In conclusion, blood cell counts revealed that 19 depending on the viral status of the fish, co-formulated products can change the cellular 20 parameters in rainbow trout, but this fact was not observed in the case of exposure to pure 21 glyphosate. 22

A potential MoA is the inhibition of AChE, demonstrated *in vitro* [19] and *in vivo* ²³ [10, 44, 46, 28, 29], which could induce oxidative stress leading to other physiological dysfunctions in cells and organisms [10, 46]. However, concentrations of glyphosate that inhibit ²⁵ AChE activity are in most cases higher than those found in the environment. Under the ²⁶ experimental conditions of this study, 96 h chemical contamination activated AChE in fish ²⁷ exposed to glyphosate and Roundup, and this tendency became statistically significant after the viral infection. This result is surprising because the majority of studies report that ²⁹ glyphosate, associated or not with co-formulants, have been shown to inhibit fish AChE 1 at concentrations ranging from 0.2 to 20 mg L^{-1} for GBH [10, 46, 28, 29] and from 1 to 2 30 mg L^{-1} for pure glyphosate [44]. However, Cattaneo et al. [10] have observed in the com-3 mon carp exposed 96 h to a GBH at a glyphosate concentration between 0.5 and $10 \,\mathrm{mg}\,\mathrm{L}^{-1}$, 4 a first inhibition phase of AChE followed by activation in the brain after 96 h of recovery. 5 An exposure of males guppies, *Poecilia vivipara*, and *Astyanax sp.* to $130 \,\mu g \, L^{-1}$ and ap-6 proximately 1 and $2 \text{ mg } \text{L}^{-1}$ of glyphosate formulated in a GBH, respectively also induced a non-statistically significant increase of AChE in the brain and/or muscle [9, 59]. Thus, it 8 could be possible that exposure to glyphosate and GBHs has an inhibitory action on ChE, 9 but that the increased levels observed result from a response of fish to counteract the effect 10 of contaminants. However, this effect of glyphosate, observed on AChE, does not appear 11 to be impacted by the Roundup formulation, but some co-formulant of Viaglif seemed to 12 counteract this effect. The activation of AChE, observed here, more probably reflects an 13 indirect effect of the contamination due to changes in some metabolic processes associated 14 with both chemical contamination and viral infection because as suggested by Payne et al. 15 [54], AChE activity is positively correlated with the global activity of fish. 16

Viral infection tends to globally decrease the levels of AChE activity in comparison to 17 levels observed post-contamination. Increased expression of AChE transcripts concomitant 18 to a reduction of a specific subunit of a nicotinic receptor of acetylcholine have been demon-19 strated in adult zebrafish, Danio rerio, exposed to a virus, with potential involvement in 20 behavioral fever [6]. However, Eder et al. [18] have shown that IHNv infection does not 21 impact levels of AChE in Oncorhynchus tshawytscha exposed or not to the herbicide chlor-22 pyrifos. Biotic or abiotic stresses have already been correlated to a loss of AChE activity 23 [31]. The infection protocol we used in this study is stressful and probably also contributed 24 to the decrease in AChE activity. 25

Several studies have reported direct or indirect effects of acute exposure of fish to ²⁶ glyphosate and some co-formulants on energy metabolism [21, 28, 29, 27, 67, 40]. Energy ²⁷ metabolism has a fundamental function in the adaptation of an organism facing multiple ²⁸ stressors, e.g. by allowing the individual to compensate for the increase in energy demand ²⁹ due to a higher maintenance cost [63]. These effects on metabolism are observed at differ-1 ent levels of organization after glyphosate exposure associated or not with co-formulants. 2 Li et al. [40] have shown a reduction in the use of glucose in goldfish, *Carassius auratus*, 3 after 96 h of exposure to a GBH, indicating a potential blockage of glycolysis and/or the 4 Krebs cycle, and thus a reduction of ATP production. This observation was associated 5 with enhanced catabolic energy production (i.e. accelerated fatty acid oxidation) and the 6 activation of creatine phosphate-based ATP regeneration. At the tissue level, the impact on 7 food absorption could be triggered by histologic damage [21]. Increased protein catabolism 8 due to higher energy demand is also suspected to be involved [29], and modifications in 9 energy storage [28], lactatemia, and glycemia [27] have been reported. Moreover, changes 10 in energy metabolism have been pointed out by proteomic analysis in the gill of the guppy, 11 *Poecilia reticulata*, exposed for 24h to a sublethal dose of GBH [66]. The authors hypoth-12 esized that inhibition of alpha-enolase observed in contaminated fish could be related to 13 alternative energy metabolisms engaged to counteract the hypoxia induced by gill structure 14 alterations. Using the same experimental design dos Santos et al. [60] showed that GBH 15 exposure increases the production of a cytochrome-c oxidase sub-unit in the liver, a key 16 organ in the detoxification process. These metabolic changes could impact the physiological 17 performances of fish, affecting biological fitness-related functions such as food intake (i.e. 18 growth), escape from predators (i.e. survival), and reproduction. These types of impacts at 19 the organism level have been observed for rainbow trout exposed to a GBH, with decreased 20 swimming performance potentially attributed to systemic impact (e.g. reduced aerobic scope 21 due to increased maintenance cost) or to a specific mechanism of toxicity (e.g. impact on 22 muscular activity) [67]. 23

No statistically significant changes in the activities of metabolic enzymes were observed ²⁴ after 96 h of contamination in our RT. In other fish species, rapid changes (i.e. 1-24h) were ²⁵ reported in response to thermal stress [31] and it is therefore probable that the concentration of glyphosate we used could have been insufficient to induce re-organization of energy ²⁷ metabolism and thus a need to adapt enzymatic activities. In silver catfish, exposed to a concentration of $0.4 \,\mathrm{mg}\,\mathrm{L}^{-1}$ of glyphosate, metabolic disruptions were expressed differently ²⁹ as a function of the tissues considered [29]. Also, sub-chronic exposure to glyphosate and GBHs at lower concentrations (i.e. $< 0.1 \,\mathrm{mg \, L^{-1}}$) up-regulated pathways implicated in en-2 ergy metabolism in the brown trout, Salmo trutta [71]. Metabolic effects in a specific tissue 3 could be masked by the fact that, in our study, we gave priority to a measure of whole-4 body response and were not able to detect tissue variations. Increased CS:CCO ratios were 5 observed in our rainbow trout after their contamination by Roundup, in accordance with 6 Pereira et al. [56] who have shown that co-formulated glyphosate at concentrations com-7 prised between 0.065 and $10 \,\mathrm{mg}\,\mathrm{L}^{-1}$ induced disruption of the mitochondrial respiratory 8 chain [32]. However, LDH:CS ratios indicate that there was not greater use of anaerobic 9 metabolism to compensate for a possible reduction in aerobic metabolism efficiency. 10

The viral challenge represents supplementary stress added to chemical contamination. 11 This context of multi-stress could generate toxic effects of glyphosate (co-formulated or not) 12 which could not be detected with the chemical contamination alone. Viral infection with 13 *IHNv* led to a rapid reduction in the activities of all the metabolic enzymes tested, except 14 for LDH in the Viaglif condition. This overall metabolic decrease could be the result of 15 prioritization of metabolic pathways essential to the anti-infectious response. Ibarz et al. 16 [32] reported a modification of the allocation of the energy and lipid metabolism in rainbow 17 trout infected by the viral hemorrhagic septicemia virus and the Gram-negative bacterium 18 Aeromonas salmonicida. This reduction could also be interpreted as an overall metabolic 19 depression resulting from the intense stress due to the viral infection, associated or not with 20 the chemical contamination [63]. After the viral infection, the enzyme CCO presented a 21 moderate but significant increase in contaminated fish that could indicate higher aerobic 22 metabolism in fish exposed to glyphosate. Moreover, this effect was not affected by the 23 presence of co-formulants. The reduced CS:CCO ratio in contaminated fish could indicate 24 that the activity in the mitochondrial matrix increased less than the activity in the membrane 25 [32]. After the viral infection, LDH activity was also increased in chemically contaminated 26 fish but this tendency was stronger for fish exposed to GBHs, indicating an increase in 27 the anaerobic metabolism potentially exacerbated by co-formulants of the GBHs. Also, the 28 slight increase of the LDH:CS ratios revealed that fish exposed to GBHs increased their use 29 of anaerobic metabolism compared to aerobic metabolism. G6PDH plays a role in protection against oxidative damage and is also considered a relevant marker of anabolism [55]. In the 2 present study, the activity of this enzyme was increased, in chemically contaminated fish 3 exposed to the virus. It may have been associated with a need to supply NADPH to maintain 4 redox homeostasis, due to both chemical and viral stresses [75]. This could also indicate that 5 anabolism was higher in contaminated fish compared to controls. TBARS levels appear to 6 be poorly informative of the oxidative stress that occurred in the exposed fish because means 7 were highly influenced by extreme values, suggesting that interfering compounds present in 8 the whole fish could have affected the assay. Information in the literature regarding levels of 9 TBARS resulting from acute exposure of fish to GBHs is quite confusing and might depend 10 on the organ, the concentration of glyphosate, and the species considered, although GBH 11 seems to be able to increase lipid peroxidation in the liver of exposed fish [27, 62, 48]. 12

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5. Conclusions

Some controversy still exists around the regulation of glyphosate based-herbicides, mainly 14 due to the integration of the effects of co-formulants in the risk assessments of these very 15 widely used products. It remains difficult to differentiate between the effects of pure 16 glyphosate and those of GBHs. This study demonstrated that acute exposure to a con-17 centration of $0.5 \,\mathrm{mg}\,\mathrm{L}^{-1}$ of glyphosate alone or associated with co-formulants in two GBHs 18 did not induce drastic toxic effects on the physiology of rainbow trout. Only limited effects 19 were observed for fish exposed to Roundup, on the red blood cell count and a metabolic 20 parameter associated with mitochondrial function. Despite this, chemical exposure does 21 not seem to alter the ability of rainbow trout to survive infection with *IHNv*. However, 22 viral infection revealed changes in specific physio-hematological parameters due to chemical 23 contamination. In particular, additional stress created by the viral infection induced differ-24 ent effects on energy metabolism pathways depending on the chemical status of the trout 25 (i.e. unexposed *versus* chemically contaminated fish). This may indicate that detoxification 26 and repair processes used to counteract the stress induced by the chemical contamination 27 represent supplementary metabolic costs that are revealed only in multistress conditons. 28 Moreover co-formulants, depending on the GBH considered, seemed to either have no impact or increased or decreased the effect of pure glyphosate. Further studies would help to clarify the mechanisms underlying the ability of glyphosate with or without co-formulants to decrease the potential of fish to face multiple stressors.

Competing interests

The authors declare that they have no conflict of interest.

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Graphical Abstract

Immunological and metabolic effects of acute sublethal exposure to glyphosate ² or glyphosate-based herbicides on juvenile rainbow trout, *Oncorhynchus mykiss* ³

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Jessy Le Du-Carrée, Joëlle Cabon, Thierry Morin, Morgane Danion



Highlights

Immunological and metabolic effects of acute sublethal exposure to glyphosate	2
or glyphosate-based herbicides on juvenile rainbow trout, Oncorhynchus mykiss	3
Jessy Le Du-Carrée, Joëlle Cabon, Thierry Morin, Morgane Danion	4
\bullet Study of acute immunotoxicity of glyphosate and co-formulated glyphosate	5
• Rainbow trout were acutely exposed and then infected with a virus	6
\bullet Acute sublet hal glyphosate exposure did not induce profound physiological changes	7
• Chemical exposure does not alter the ability of rainbow trout to survive viral infection	8
• Environmental stresses could trigger the effects of chemical exposure	9

Graphical Abstract

Immunological and metabolic effects of acute sublethal exposure to glyphosate ² or glyphosate-based herbicides on juvenile rainbow trout, *Oncorhynchus mykiss* ³

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Jessy Le Du-Carrée, Joëlle Cabon, Thierry Morin, Morgane Danion

