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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 glyphosate-based 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 µg 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-6-phosphate 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 goods and services to human societies as a source of both food and water [4]. Water quality 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, are a particular threat to their integrity and this trend could be increased in a context of global climate change, where different biotic and abiotic stressors might interact [74]. Ecotoxicology aims to evaluate the impact of these chemical contaminants on ecosystems and to define regulatory limits that maintain the use of these compounds under sustainable levels [5]. Glyphosate is the active substance (AS) of glyphosate-based herbicides (GBHs), which are the most commonly used pesticides worldwide. Concerns have been raised about its environmental safety [69, 68] and re-registration of this compound and its associated commercial products is still the subject of debate at the level of the European Union [64]. While glyphosate shows low potential for bioaccumulation in animal tissues [22], low levels of this chemical are ubiquitously encountered in the environment and aquatic organisms are therefore continuously exposed. In France, from 2007 to 2017, glyphosate detection in surface water increased from 22.2 to 49.7% of sampling points analyzed, and the mean maximum annual concentrations quantified during this interval among all the sampling points ranged

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from 2.4 to 70.2 µg 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 µg 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 µg 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 biological organization. Acute exposure is defined as contact between the organisms (fish) used as indicators and the chemical to be tested, less than or equal to 96 hours [52]. This approach is rapid and convenient to define the dose inducing 50% mortality (LC50) in a specific species [51]. $LC50_{96\,h}$ values comprised between 22 and > 1000 mg L^{-1} for the AS and comprised between 4.2 and 52 mg L^{-1} for a GBH were determined for several fish species, including several Salmonidae [26]. In rainbow trout (RT), one of the most important farmed fish species in Europe and a relevant model in ecotoxicology, EFSA [22] recently reported an LC50 value of 38 mg L^{-1}. This LC50 value is at least about 362 times higher than the PEC calculated by EFSA, and acute exposure to glyphosate is therefore unlikely to have a significant environmental impact. For the present study, we chose a concentration of AS 76 times lower than the LC50 reported by European Food Safety Authority [22] that would make it possible to detect changes specific to the glyphosate mode of action (MoA), rather than non-specific disruptions induced by high toxic stress. However, several studies have suggested that sublethal doses of glyphosate, administered as the AS or GBH, could induce biological or physiological alterations which might affect wild fish living in a complex and stressful environment [38, 41, 46, 23, 28, 53], impacting their thermal tolerance [76] or pathogen susceptibility [39]. Immune toxicity of glyphosate has been demonstrated in certain *in vitro* assays [20, 70]. Moreover, the fish immune system seems to be particularly influenced by exposure to pure glyphosate AS [42, 71, 45] or to GBHs [37, 23, 38, 47]. However, studies linking disruptions of hemato-immunological parameters to pathogen susceptibility of exposed fish are still lacking. Energy metabolism is another critical function involved in the adaptation of an organism facing multiple stressors, e.g. by compensating for the

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 (Mean ± standard deviation = 2.8 g ± 0.8 g) from the protected and monitored fish facilities of the ANSES Plouzané Laboratory site (France) were used. The fish growth period was conducted in 12 tanks (30 L) positioned in a confined room, supplied with an open circuit with filtered river water and equipped with adapted aeration to maintain oxygen levels between 6 and 8 mg L^{-1}. Every morning, after fish care and tank maintenance, trout were fed with trout-specific food (Neo supra AL4, Le Gouessant®), at 1.5% of biomass. The same photoperiod (12 h of daylight) was maintained throughout the experiment. Water temperature during the chemical exposure was maintained at 15 °C±2 °C, while during the viral challenge water temperature was set to 11 °C±2 °C to ensure a range within the thermal optimum of the virus.

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

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

2.3. Virus production and titration

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

2.4. Experimental design

2.4.1. Ethics statement

All animal studies were carried out in the approved infrastructure of the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) at Plouzané (France; approval number D29-212-03), in strict accordance with the European guidelines and recommendations on animal experimentation and welfare (European Union Directive 2010/63). All animal experimental procedures were analyzed 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#2018020115216522. Euthanasia involved the addition of a lethal dose of 100 ppm of Eugenol into the tank water.

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 semi-static river water, injecting a volume of 75 mL of each concentrated solution to obtain a final theoretical exposure concentration of 500 µg L^{-1} of glyphosate (1b). Every 24 hours, contaminated water was entirely renewed and chemical contamination was reproduced.

2.4.3. Viral challenge

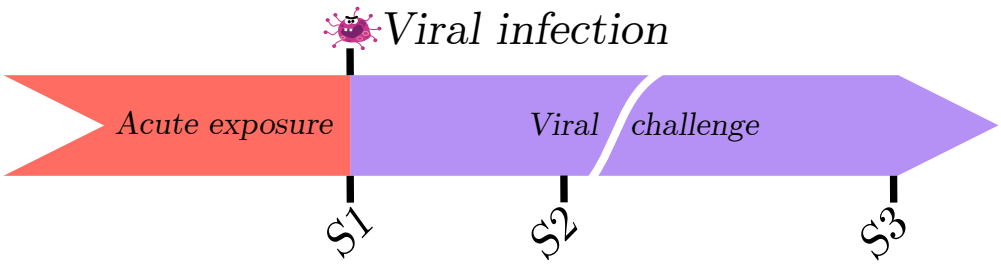
After 96 h of chemical exposure, 99 fish for each chemical treatment (i.e. 33 fish per replicate of a condition) were randomly distributed in four 10 L tanks with constant water renewal (i.e. three tanks were infected with the *IHNv* and one was used as the uninfected viral control; Figure 1b). Infection 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 $TCID_{50}$ 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 at −20 °C for viral examination.

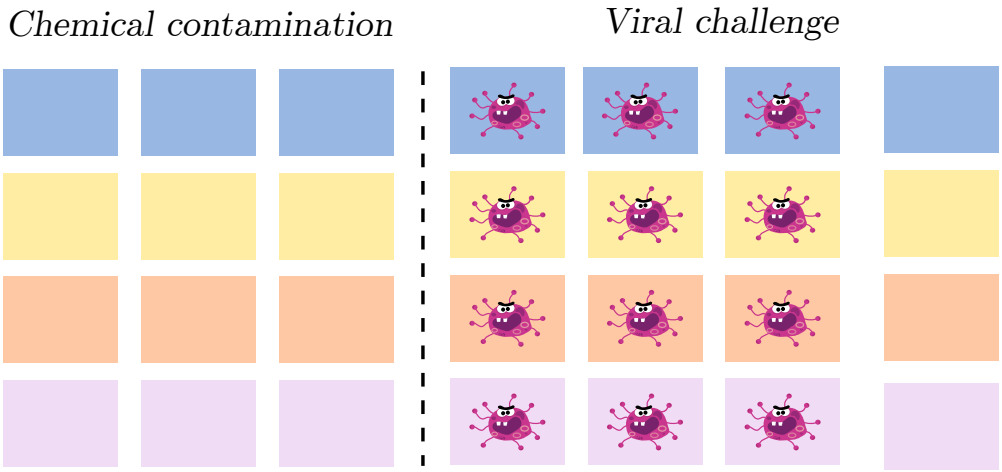
2.5. Samples and sampling date

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

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



(a)



(b)

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). For each fish, a blood sample was taken by withdrawing blood from the caudal vein with a lithium heparin hematocrit tube (Greiner ref. KG454244). At S1 and S2, 5 µL of whole blood was used for hematological analyses. At S3, 50 µL of whole blood was sampled and then centrifuged ($1,200 \times g$, 10 min, 4 °C) and only plasma was stored at −80 °C for serological analysis. At S1 and S2, whole fish bodies were flash-frozen in liquid nitrogen and stored at −80 °C for future analyses.

2.6. Chemical and biological parameters

2.6.1. Chemical parameters

Quantification of glyphosate using direct competitive ELISA assay (Novakits, ref. 1500086) was done before and after the water renewal, following the supplier's instructions. Briefly, the river water was first filtered at 0.2 µm (Clearline ref. 146560), 250 µL of the filtrate was derivatized with 100 µL of the derivation solution provided in the kit. Then, 50 µL was transferred to wells in a microplate coated with goat anti-rabbit antibodies and 50 µL of the reagent containing rabbit anti-glyphosate antibody was added. A competitive reaction for binding sites (between glyphosate and glyphosate enzyme conjugate) was started by the addition of 50 µL of enzyme conjugate solution for 1 h. After a washing step using 3×250 µL, 150 µL of the substrate solution was added into the wells and the colored reaction was stopped after 25 min by adding 100 µL of the stop solution. Plates were read at 450 nm on a TECAN Spark 10M microplate spectrophotometer. The development of color is inversely proportional to the concentration of glyphosate in the sample. Standard curves were generated using the four-parameter log-logistic function, LL.4, of the R package "drc" [61]. 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 aminomethylphosphonic acid (AMPA) by HPLC/fluorometric methods (Method ref. ANA-I10.MOA.69.B) was done by an external provider (Labocea, France) only after the first water renewal and recontamination, in one tank per condition.

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 was then diluted to 10^{-11}, and the virus concentration was determined for each fish, as described in section 2.3.

2.6.3. Immune parameters

At S1 and S2, counting of red and white blood cells was performed on a Thoma cell hemocytometer using whole blood diluted to 1/200 in Giemsa solution [35]. The alternative 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 semi-quantification of anti-*IHNv* antibodies in the plasma of surviving fish were performed using a modification of the procedure of Jorgensen et al. [33] described by Dupuy et al. [17].

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

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

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

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 were produced using the ggplot2 package [73]. 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 [16]. In the case of normal and heteroscedastic data, modified one-way Anova tests were used to compare means [72], followed by a post-hoc test of Tamhane-Dunnett [50]. In the case of non-normal data, a Kruskal-Wallis test was used to compare means, followed by a post-hoc test of Dunn [15]. Differences between proportions of seropositive individuals between chemical treatments were compared using 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 correlation of Pearson. Mortality rates for the different chemical treatments were compared using the "survival" package [65] by following the procedure described by Doumayrou et al. [14]. Restricted mean survival time, an integrated parameter representing both the dynamics and the intensity of mortality [65], was calculated using the "survival" package.

3. Results

3.1. Determination of glyphosate concentrations during acute contamination

Glyphosate and AMPA were not detected in control tanks, and AMPA was not detected in any of the contaminated samples analyzed. Concentrations of glyphosate were between 427.82 and 497.77 µg L^{-1} for all conditions in the contaminated tanks at the end of the first 24 hours of contamination, but also after water renewal and re-contamination using an ELISA assay, except for the Roundup condition where a higher concentration (728.35 µg L^{-1}) was detected after re-contamination. Quantifications done by HPLC yielded similar concentrations, between 428.80 and 477.00 µg L^{-1}. Regardless of the method considered and excluding the value of 728.35 µg L^{-1} obtained using ELISA for the Roundup condition, glyphosate con-

centrations obtained in contaminated tanks did not show a difference greater than 15% from the expected values (i.e. 500 µg L^{-1}). (Table 1).

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

Sampling	**Condition**	**Glyphosate concentration** (µg L^{-1})	
		ELISA	HPLC
After 24h of exposure	**C**	nd	nd
	G	460.48 ± 77.92	nm
	R	497.77 ± 11.55	nm
	V	427.82 ± 4.45	nm
After water renewal and recontamination	**G**	464.48 ± 53.15	477
	R	728.35 ± 12.15	466
	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. During the viral challenge, while no mortality occurred in the non-infected control fish, mortality in infected tanks began overall at 4 or 5 dpi. At 60 dpi, cumulative mortality (mean ± se, n=3) reached 61.8 ± 0.9% for the control, 60.4 ± 6.2% for glyphosate, 64.9 ± 2.2% for Roundup, and 67.8 ± 4.5% for Viaglif (Figure 2), without significant differences in mortality between groups. Slightly faster and higher mortality was observed in fish exposed to Roundup and Viaglif, with shorter restricted mean survival times (RMST) of (mean ± se, n = 3) 33 ± 1.0 and 31 ± 2.6 dpi respectively, compared to 35 ± 0.7 and 36 ± 3.0 dpi for the control and glyphosate conditions.

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 between 12 and 13 dpi particularly for Roundup and to a lesser extent for the control and 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.

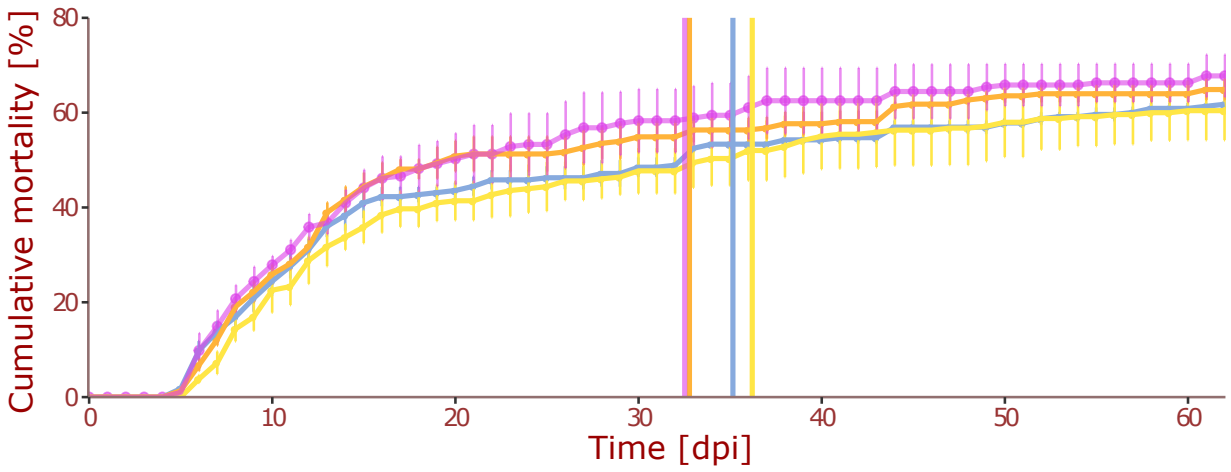


Figure 2: Kinetics of cumulative mortality in fish exposed to chemical contamination and infected with *IHNv*. Data are expressed as a function of time in days post-infection (dpi) for the glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue) groups. Error 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 the two GHBs compared to the control, particularly for fish exposed to Roundup with a significant rise of 35% ($p < 0.05$; Figure 3a). WBCCs did not vary significantly, regardless of the chemical contamination (Figure 3b).

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

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 ± standard-error, n = 3).

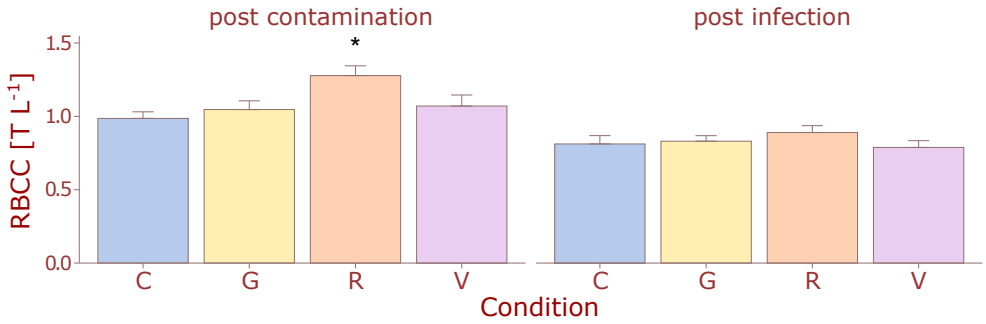
Parameter	**Condition**			
	C	G	R	V
Proportion of *IHNv*-positive fish	3/3	2/3	3/3	1/3
***IHNv* titer**	4.86×10^4 (4.37×10^4)	7.12×10^4 (6.48×10^4)	4.58×10^5 (4.51×10^5)	6.32×10^5

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

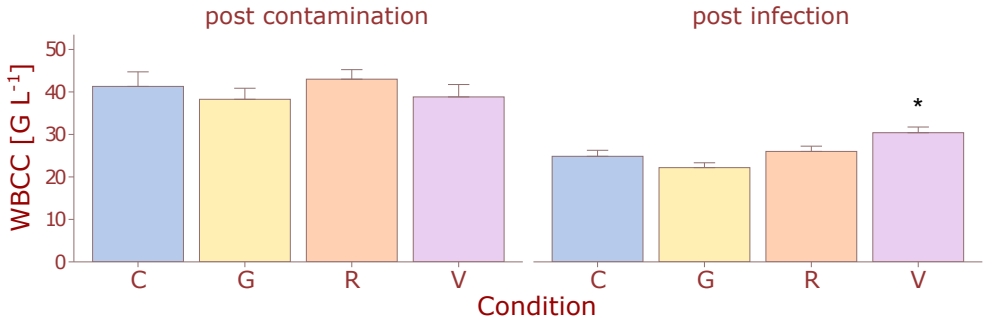
At 60 dpi (S3), similar proportions from 67 to 80% of the survivor fish analyzed by condition were seropositive for *IHNv* (Table 2). The control and glyphosate conditions were characterized by percentages of highly *IHNv* seropositive fish (i.e. presenting an antibody titer greater than 640), which 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).

3.4. Oxidative stress and metabolic parameters in whole fish

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



(a) RBCC



(b) WBCC

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 ($20 \leq n \leq 33$). 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 ± 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 ≥ 640 and the total number of seropositive fish. Mean antibody titers were calculated for highly seropositive fish only.

Parameter	**Condition**			
	C	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-*IHNv* antibody titer	1189 (259)	914 (129)	2240 (656)	1280 (405)

chemical exposure conditions taken together), a moderate correlation was observed between LDH activity and mortality (Pearson's correlation = 0.55, p = 0.07). The ratio between LDH and CS activities presented high variability, regardless of the chemical condition and the 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 chemical contamination, and between 611 and 777% 96 h after the viral infection. The ratio between CS and CCO activities was significantly higher in fish contaminated by Roundup after 96 h of chemical exposure ($p < 0.05$) compared to the other conditions (128% for the control compared to 150% for the Roundup condition, a difference of 17%). After the viral infection, this parameter presented differences of 26, 27, and 32% for glyphosate, Roundup and Viaglif compared to the control ($p < 0.05$), respectively. Ratios were comprised between 121 and 136% for chemically contaminated fish and equal to 190% for the controls. G6PDH activities in infected fish exposed to glyphosate and GBHs were increased compared to the controls. This tendency was particularly marked in GBH conditions, with observed differences of 126 and 138% for Roundup and Viaglif ($p < 0.05$), respectively (4). TBARS

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

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

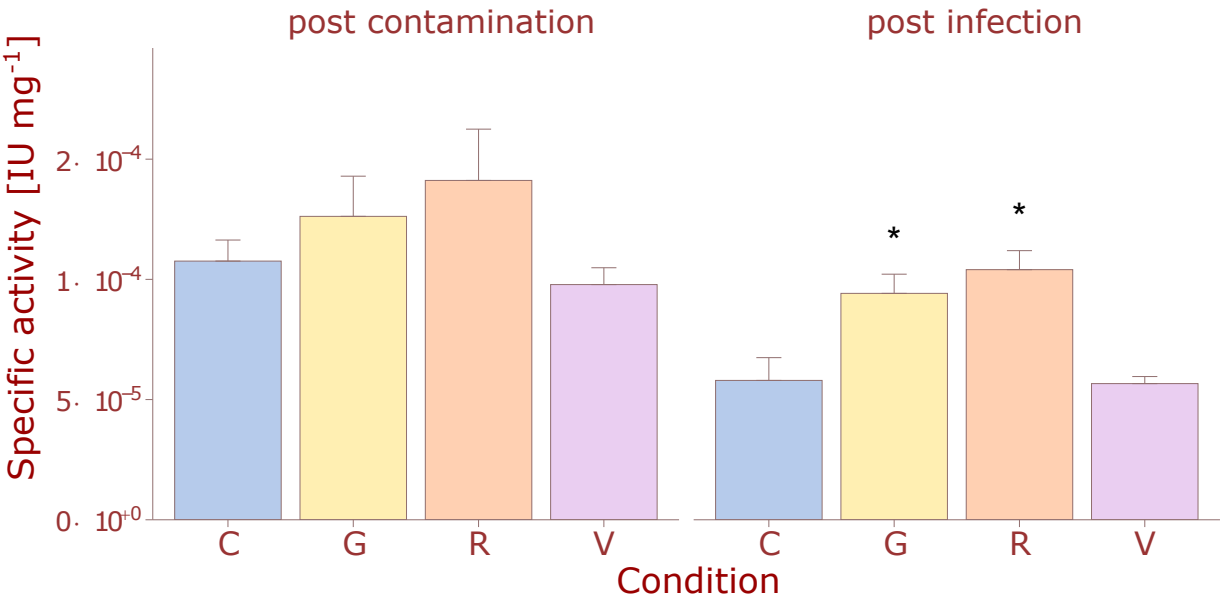


Figure 4: Mean specific enzymatic activities of AChE in fish exposed to glyphosate (G, in yellow), Roundup (R, in orange), Viaglif (V, in purple) and control (C, in blue) at S1 (post contamination) and S2 (post infection). Enzymatic activity is expressed in IU mg^{-1} of protein. Standard errors are represented at the top of each bar ($n = 15$ for sample post contamination and $8 \leq n \leq 15$ 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 ($9 \leq n \leq 15$ at S1 and $7 \leq n \leq 15$ 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**				
		CS	CCO	LDH	G6PDH	TBARS
post conta.	**C**	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)
	R	0.41	0.27	2.25	0.016	0.88
		(0.021)	(0.011)	(0.43)	(0.0016)	(0.36)
	V	0.33	0.28	1.57	0.014	0.71
		(0.021)	(0.015)	(0.083)	(0.00099)	(0.31)
post inf.	**C**	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)
	R	0.32	**0.24***	2.03	**0.01***	0.84
		(0.023)	(0.017)	(0.2)	(0.0013)	(0.45)
	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.

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

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.

The innate and specific immune systems play a fundamental role in intensive aquaculture and the natural environment where fish are threatened by a large diversity of pathogens [11].

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 organization, such as the molecular [42, 71] or cellular levels [45, 37, 23, 38, 47], only few data are available on the effects of glyphosate on overall immune system functioning. Nonetheless, studies using pathogens with direct fish infections after a period of chemical exposure are particularly useful to detect the immunotoxic effects of chemical contaminants [58]. To our knowledge, no studies using viral challenge after exposure to glyphosate or GBHs are available in the literature. Kreutz et al. [39] have already shown that acute exposure of silver catfish fingerlings, *Rhamdia quelen*, to 730 µg L^{-1} of a GBH induced changes in immune cells and consequently higher susceptibility to the bacterium *Aeromonas hydrophila*.

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

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

A potential MoA is the inhibition of AChE, demonstrated *in vitro* [19] and *in vivo* [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 AChE activity are in most cases higher than those found in the environment. Under the experimental conditions of this study, 96 h chemical contamination activated AChE in fish 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

glyphosate, associated or not with co-formulants, have been shown to inhibit fish AChE at concentrations ranging from 0.2 to 20 mg L^{-1} for GBH [10, 46, 28, 29] and from 1 to 30 mg L^{-1} for pure glyphosate [44]. However, Cattaneo et al. [10] have observed in the common carp exposed 96 h to a GBH at a glyphosate concentration between 0.5 and 10 mg L^{-1}, a first inhibition phase of AChE followed by activation in the brain after 96 h of recovery. An exposure of males guppies, *Poecilia vivipara*, and *Astyanax sp.* to 130 µg L^{-1} and approximately 1 and 2 mg 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 could be possible that exposure to glyphosate and GBHs has an inhibitory action on ChE, but that the increased levels observed result from a response of fish to counteract the effect of contaminants. However, this effect of glyphosate, observed on AChE, does not appear to be impacted by the Roundup formulation, but some co-formulant of Viaglif seemed to counteract this effect. The activation of AChE, observed here, more probably reflects an indirect effect of the contamination due to changes in some metabolic processes associated with both chemical contamination and viral infection because as suggested by Payne et al. [54], AChE activity is positively correlated with the global activity of fish.

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

Several studies have reported direct or indirect effects of acute exposure of fish to glyphosate and some co-formulants on energy metabolism [21, 28, 29, 27, 67, 40]. Energy metabolism has a fundamental function in the adaptation of an organism facing multiple stressors, e.g. by allowing the individual to compensate for the increase in energy demand

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

No statistically significant changes in the activities of metabolic enzymes were observed after 96 h of contamination in our RT. In other fish species, rapid changes (i.e. 1-24h) were 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 metabolism and thus a need to adapt enzymatic activities. In silver catfish, exposed to a concentration of $0.4\,\mathrm{mg\,L^{-1}}$ of glyphosate, metabolic disruptions were expressed differently

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 energy metabolism in the brown trout, *Salmo trutta* [71]. Metabolic effects in a specific tissue could be masked by the fact that, in our study, we gave priority to a measure of whole-body response and were not able to detect tissue variations. Increased CS:CCO ratios were observed in our rainbow trout after their contamination by Roundup, in accordance with Pereira et al. [56] who have shown that co-formulated glyphosate at concentrations comprised between 0.065 and $10\,\mathrm{mg\,L^{-1}}$ induced disruption of the mitochondrial respiratory chain [32]. However, LDH:CS ratios indicate that there was not greater use of anaerobic metabolism to compensate for a possible reduction in aerobic metabolism efficiency.

The viral challenge represents supplementary stress added to chemical contamination. This context of multi-stress could generate toxic effects of glyphosate (co-formulated or not) which could not be detected with the chemical contamination alone. Viral infection with *IHNv* led to a rapid reduction in the activities of all the metabolic enzymes tested, except for LDH in the Viaglif condition. This overall metabolic decrease could be the result of prioritization of metabolic pathways essential to the anti-infectious response. Ibarz et al. [32] reported a modification of the allocation of the energy and lipid metabolism in rainbow trout infected by the viral hemorrhagic septicemia virus and the Gram-negative bacterium *Aeromonas salmonicida*. This reduction could also be interpreted as an overall metabolic depression resulting from the intense stress due to the viral infection, associated or not with the chemical contamination [63]. After the viral infection, the enzyme CCO presented a moderate but significant increase in contaminated fish that could indicate higher aerobic metabolism in fish exposed to glyphosate. Moreover, this effect was not affected by the presence of co-formulants. The reduced CS:CCO ratio in contaminated fish could indicate that the activity in the mitochondrial matrix increased less than the activity in the membrane [32]. After the viral infection, LDH activity was also increased in chemically contaminated fish but this tendency was stronger for fish exposed to GBHs, indicating an increase in the anaerobic metabolism potentially exacerbated by co-formulants of the GBHs. Also, the slight increase of the LDH:CS ratios revealed that fish exposed to GBHs increased their use

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 present study, the activity of this enzyme was increased, in chemically contaminated fish exposed to the virus. It may have been associated with a need to supply NADPH to maintain redox homeostasis, due to both chemical and viral stresses [75]. This could also indicate that anabolism was higher in contaminated fish compared to controls. TBARS levels appear to be poorly informative of the oxidative stress that occurred in the exposed fish because means were highly influenced by extreme values, suggesting that interfering compounds present in the whole fish could have affected the assay. Information in the literature regarding levels of TBARS resulting from acute exposure of fish to GBHs is quite confusing and might depend on the organ, the concentration of glyphosate, and the species considered, although GBH seems to be able to increase lipid peroxidation in the liver of exposed fish [27, 62, 48].

5. Conclusions

Some controversy still exists around the regulation of glyphosate based-herbicides, mainly due to the integration of the effects of co-formulants in the risk assessments of these very widely used products. It remains difficult to differentiate between the effects of pure glyphosate and those of GBHs. This study demonstrated that acute exposure to a concentration of $0.5\,\mathrm{mg\,L^{-1}}$ of glyphosate alone or associated with co-formulants in two GBHs did not induce drastic toxic effects on the physiology of rainbow trout. Only limited effects were observed for fish exposed to Roundup, on the red blood cell count and a metabolic parameter associated with mitochondrial function. Despite this, chemical exposure does not seem to alter the ability of rainbow trout to survive infection with *IHNv*. However, viral infection revealed changes in specific physio-hematological parameters due to chemical contamination. In particular, additional stress created by the viral infection induced different effects on energy metabolism pathways depending on the chemical status of the trout (i.e. unexposed *versus* chemically contaminated fish). This may indicate that detoxification and repair processes used to counteract the stress induced by the chemical contamination represent supplementary metabolic costs that are revealed only in multistress conditons.

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 or glyphosate-based herbicides on juvenile rainbow trout, *Oncorhynchus mykiss*

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

Juvenile rainbow trout

Glyphosate or GBHs exposure (500 µg/L for 96h)

No mortality
↗ RBCC
= WBCC
= AChE
= TBARs
= CS, CCO, LDH, G6PDH

IHNv infection

No excess mortality (~60%)
= RBCC
↗ WBCC in GBHs
↗ AChE
= TBARs
↗ CCO, LDH, G6PDH

Highlights

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- Study of acute immunotoxicity of glyphosate and co-formulated glyphosate
- Rainbow trout were acutely exposed and then infected with a virus
- Acute sublethal glyphosate exposure did not induce profound physiological changes
- Chemical exposure does not alter the ability of rainbow trout to survive viral infection
- Environmental stresses could trigger the effects of chemical exposure

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