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ABSTRACT

Pharmaceuticals are emerging pollutants of concern for aquatic ecosystems where they are occurring in complex mixtures. In the present study, the chronic toxicity of an environmentally relevant pharmaceutical mixture on juvenile rainbow trout (*Oncorhynchus mykiss*) was investigated. Five pharmaceuticals (paracetamol, carbamazepine, diclofenac, naproxen and irbesartan) were selected based on their detection frequency and concentration levels in the Meuse river (Belgium). Fish were exposed for 42 days to three different concentrations of the mixture, the median one detected in the Meuse river, 10-times and 100-times this concentration. Effects on the nervous, immune, antioxidant, and detoxification systems were evaluated throughout the exposure period and their response standardized using the Integrated Biomarker Response (IBRv2) index. IBRv2 scores increased over time in the fish exposed to the highest concentration. After 42 days, fish exposed to the highest concentration displayed significantly higher levels in lysozyme activity ($p < 0.01$). The mixture also caused significant changes in brain serotonin turnover ($p < 0.05$). In short, our results indicate that the subchronic waterborne exposure to a pharmaceutical mixture commonly occurring in freshwater ecosystems may affect the neuroendocrine and immune systems of juvenile rainbow trout.

1. Introduction

Due to their constantly growing consumption, biological activity and continuous release, pharmaceuticals' occurrence in freshwater ecosystems is a raising concern (Godoy and Kummrow, 2017; Hughes et al., 2013). Wastewater treatment plants (WWTP) are one of the largest sources of pharmaceuticals discharge in surface water along with livestock farms and domestic wastes (Boxall et al., 2012; Sim et al., 2011; Zhang et al., 2008). As a result, more than 200 different pharmaceutical compounds have been detected in European rivers in concentrations ranging from a few ng L^{-1} to several μg L^{-1}. Even though individual pharmaceuticals are unlikely to cause acute toxic effects at these low concentrations, the chronic exposure to complex mixtures encompassing different drug classes with various modes of action can cause sublethal effects to non-target organisms (Cleuvers, 2003; Li and Lin, 2015; Quinn et al., 2009). These effects are currently overlooked by the risk assessment methods usually employed, which focus on individual compound toxicity (Hook et al., 2014).

Mixture toxicity assessment studies undertaken so far were mostly carried out on organisms of low trophic levels such as algae (DeLorenzo

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and Fleming, 2008; Geiger et al., 2016) or crustacean (Cleuvers, 2003; Nieto et al., 2016). However, fish tend to display a higher sensitivity to pharmaceuticals exposure as pharmaceuticals' biological targets have been highly evolutionary conserved in this taxonomic group compared to other aquatic organisms (Brown et al., 2014; Henschel et al., 1997).

In this context, the present study aimed to assess the subchronic toxicity of a cocktail of 5 pharmaceuticals (carbamazepine, irbesartan, paracetamol, naproxen, and diclofenac) at 1, 10, and 100 times their measured median concentration in the Meuse river (Belgium), on juvenile rainbow trout (*Oncorhynchus mykiss*). The selection of these molecules and their concentration levels were based on the results obtained during a large sampling and analytical study conducted between 2015 and 2016 in the entire anthropic water cycle (Nott et al., 2018). The selected compounds belong to different classes of pharmaceuticals (neuroleptic, antihypertensive, analgesic and nonsteroidal anti-inflammatory drugs (NSAIDs)) which differ by their mode of action (MOA) and biological targets (Fent et al., 2006). Therefore, multiple toxicological endpoints representative of the antioxidant and detoxification mechanisms as well as the immune and nervous functions were studied. This multi-biomarker approach allows establishing a robust diagnosis of the consequences of pharmaceutical mixture's exposure to fish health and fitness and not only their effect on specific physiological functions. However, it also makes it more challenging to draw a global conclusion regarding the risk represented by the mixture. Therefore, to facilitate their interpretation, biomarker responses were standardized using the Integrated Biomarker Response (IBRv2) index developed by Sanchez et al. (2013). This index is employed to assess organisms' stress status in both field and laboratory experiments, and to point out biomarker response patterns representative of a certain type of contamination (Ferreira et al., 2015; Serafim et al., 2012).

2. Materials and methods

2.1. Pharmaceutical mixture

Carbamazepine 98% (CAS 298464, Alfa Aesar), paracetamol 98% (CAS 103902, VWR), diclofenac sodium salt 98% (CAS 15307796, Sigma-Aldrich), naproxen sodium 98% (CAS 26159342, VWR) and irbesartan 98% (CAS 138402116, Sigma-Aldrich) were used for the experiment.

The mixture stock solution (20,000,000×) was prepared in dimethylsulfoxide (DMSO) and diluted in water to obtain the working solutions (2000×).

The experiment was carried out using three different concentrations of the mixture, 1-fold (1×,) 10-fold (10×), and 100-fold (100×) the median environmental concentration (Table 1). A peristaltic pump (Watson Marlow) supplied the mixture from daily renewed working solutions at a rate of 100 μL min^{-1}. The water flow was adjusted to 200 mL min^{-1} to ensure constant water renewal. Four tanks were implemented for each concentration level. Four additional tanks were used as controls. In those tanks, fish were exposed to the volume of DMSO used for the 100X concentration (v/v, 0.002%).

The concentration of each compound was checked weekly by sampling 300 mL (for the control and 1× tanks) or 20 mL (for the 10× and 100× tanks) of water in each tank. For the control and 1× tanks, the compounds were extracted by solid phase extraction on OASIS HLB cartridges and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). For the 10× and 100× tanks, the samples were directly analyzed by LC-MS/MS. Further details on these analyses can be found in Schmitz et al. (2018). Targeted and measured concentrations of each compound are indicated in Table 1. Except for paracetamol which displayed degradation, the pharmaceutical concentrations measured in the tanks remained stable over the experiment and ranged from 80% to 120% of the expected ones.

2.2. Fish and rearing conditions

Investigations were carried out at the University of Namur (Namur, Belgium) according to the guidelines for animal use and care in compliance with the Belgian and European regulations on animal welfare (ethical protocol KE17/290). Rainbow trout juveniles (25–35 g body weight, 5-month-old) were provided by a trout farm (Pierru fish farm, Rogny-Les-Moulins, France) and transferred to the UNamur's experimental facilities. Fish were distributed into 16 glass aquaria (volume = 100 L, 35 fish per aquarium, 4 aquaria per condition) at 13 °C under a constant photoperiod (12L:12D). Fish were acclimatized for 10 days before the beginning of the experiment and were fed daily with commercial dry pellets (2% biomass) (Troco Supreme, Coppens). Physicochemical data for water quality control were measured daily with a multiparameter probe (WTW, Multi 350i) in the outlet pipe: dissolved oxygen (O_2): 8.3 ± 0.2 mg L^{-1}; pH: 7.6 ± 0.03; temperature: 13.4 ± 0.2 °C; N-NO_2-: 0.048 ± 0.01 mg L^{-1}; NH_4^+: 0.19 ± 0.5 mg L^{-1}.

2.3. Sampling procedures

Fish (4 per tank) were randomly sampled at days 1, 7, 21, and 42 for biomarker analysis. Following anesthesia with MS-222 (150 mg L^{-1}, Sigma-Aldrich), blood was collected within 5 min by caudal vein puncture using a sterile 1 mL heparinized syringe. Plasma was separated by centrifugation (4 °C, 10,000×*g*, 10 min) and stored at −80 °C prior to analysis. Fish were then euthanized by cervical dislocation and the liver, gonads, brain, and muscle tissues were collected, frozen in liquid nitrogen and stored at −80 °C until assayed. The spleen was sampled and kept on ice for immediate analysis.

2.4. Fish growth parameters and general condition

Following euthanasia, the wet weight of each sampled fish was measured. The mean Specific Growth Rate (SGR) was determined on day 42 for each experimental condition and was calculated as follows:

$$SGR \quad (\% \quad day^{-1}) = \left(\frac{\ln(W_2) - \ln(W_1)}{t_2 - t_1}\right) X \quad 100$$

With W_2 and W_1 being the average weight of the fish on day 42 and day 1, t_2 and t_1 the number of experimentation days between the first and the

Table 1
Targeted and measured concentrations (μg L^{-1}) of paracetamol, irbesartan, carbamazepine, naproxen, and diclofenac for Control (DMSO, v/v, 0.002%), 1× (mean environmental concentrations in the Meuse river), 10× and 100× tanks (mean ± SD).

		Paracetamol	Irbesartan	Carbamazepine	Naproxen	Diclofenac
Targeted	Control	0	0	0	0	0
	1×	0.1	0.05	0.025	0.025	0.025
	10×	1	0.5	0.25	0.25	0.25
	100×	10	5	2.5	2.5	2.5
Measured	Control	0.01	0	0	0	0
	1×	0.08 ± 0.3	0.048 ± 0.0	0.026 ± 0.0	0.022 ± 0.0	0.027 ± 0.00
	10×	0.6 ± 0.0	0.43 ± 0.0	0.25 ± 0.0	0.26 ± 0.0	0.26 ± 0.0
	100×	4.0 ± 0.5	4.7 ± 0.2	2.8 ± 0,1	3.1 ± 0.1	2.9 ± 0.1

last sampling day.

2.5. Bioconcentration of contaminants in fish

Fish (n = 4) were randomly sampled in each tank at days 7, 21 and 42 for bioconcentration analysis. Briefly, internal standards were spiked to 5 g of whole fish homogenized samples. After elimination of the proteins by precipitation, the pharmaceuticals were extracted by solid phase extraction on OASIS HLB cartridges and analyzed by UHPLC-MS/MS. The complete protocols for sample preparations and analyses are described in Schmitz et al. (2018).

2.6. Immunotoxicity biomarkers

2.6.1. Plasma lysozyme activity

Plasma lysozyme activity was measured based on the turbidimetric method (Ellis, 1999). Briefly, 250 μL of a *Micrococcus lysodeikticus* suspension (0.6 mg L^{-1} in phosphate buffer, pH 6.2) were added to 10 μL of plasma. Change in absorbance at 450 nm was recorded during 15 min. The activity was expressed as the amount of enzyme causing a decrease of the optical density of 0.001 min^{-1}. The measurements were carried out in triplicates.

2.6.2. Alternative complement pathway (ACH50)

The alternative complement pathway was assayed according to Sunyer and Tort (1995). Briefly, 10 μL of a rabbit red blood cells (RRBC) suspension (3% in veronal buffer, Biomerieux) were added to plasma serial dilutions. ACH50 value was defined as the reciprocal of the plasma dilution which induces 50% of RRBC hemolysis. Positive (100% hemolysis) and negative (0% hemolysis) controls were respectively obtained by adding 60 μL of either distilled water or veronal buffer to the 10 μL of the RRBC solution. Following incubation (2 h, 20 °C), samples were centrifuged (200×*g*, 5 min, 4 °C) and the supernatant absorbance was read at 405 nm. The measurements were carried out in duplicates.

2.7. Hepatic oxidative stress and detoxification biomarkers

Livers were ground with a bead homogenizer (FastPrep-24 5G, MP Biomedicals) in phosphate buffer pH 7.8 and centrifuged (10,000×*g*, 15 min, 4 °C). The supernatant was collected and stored at −80 °C for biomarker analysis. Glutathione-*S*-transferase (GST), ethoxyresorufin-O-deethylase (EROD), glutathione peroxidase (GPx), catalase (CAT), total glutathione (GSH) assays were carried out according to Babo and Vasseur (1992), Flammarion et al. (1998), Habig et al. (1974), Paglia and Valentine (1967) methods. Data were normalized to the protein content with the Bradford (1976) method. Analyses were performed in duplicates using a fully automated liquid handling robot (EVO 150, Tecan) and a microplate reader (Synergy H4, BioTek Instruments).

2.8. Neurotoxicity biomarkers

2.8.1. Brain neurotransmitters

Brain serotoninergic and dopaminergic activities were assessed using HPLC according to Lepage et al. (2000) method with some modifications. Brains were pooled equally using the tanks as experimental unit (4 fish per pool) and homogenized using a Bullet Blender Storm 24 (NextAdvance) in tubes containing 0.5 mm zirconium oxide beads (Dutscher) and 1.5 mL per g of tissue of mobile phase used for the chromatography. They were then sonicated, centrifuged (20,000×*g*, 10 min, 4 °C), and filtered through 0.5 μm filters (Phenomenex). Proteins were eliminated by adding methanol to the filtrate (v/v, 3:2). After centrifugation (15,000×*g*, 20 min, 4 °C), the supernatant was transferred into a new vial to be concentrated in a Speed Vac (Jouan) to reach a final volume of 250 μL. The homogenates were filtered again, and the filtrate injected into the HPLC system. Analyses were performed using a GP50 gradient pump (Dionex) equipped with an autosampler FAMOS (LC packings). Neurotransmitters were monitored using a DC amperometry detector (Dionex) with Glassy Carbon Working Electrode (0.700 V, Ag/AgCl – P/N 061677). Chromeleon™ software (6.8) (Dionex) was used for data acquisition and processing. The samples were individually applied at 1 m min^{-1} on a 2.6 μm particle size (150 × 4.6 mm, I.D.) C_{18} analytical Kinetex column maintained at 25 °C. The mobile phase consisted of 65 mM NaH_2PO_4, 1.63 mM octane sulfonic acid (OSA, Sigma-Aldrich), 0.1 mM EDTA-Na_2 and 13% MeOH adjusted to pH 2.8 with orthophosphoric acid. Purified hormones (Sigma-Aldrich) were used for standard solutions. The intra- and inter-assay coefficients of variation for the tested hormones were under 5.9% and 7.4%, respectively. Finally, serotoninergic and dopaminergic activities were expressed as hydroxyl-indol-acetic acid (5-HIAA)/serotonin (5-HT) and 3,4-dihydroxyphenylacetic acid (DOPAC) / dopamine (DA) ratios respectively.

2.8.2. Muscle acetylcholinesterase (AchE) activity

Muscle tissues were homogenized using a Bullet Blender Storm 24 (NextAdvance) in tubes containing 0.5 mm zirconium oxide beads (Dutscher) and cold 0.1 M phosphate buffer (pH 7.5) containing protease inhibitors (v/v; 1:5). The homogenates were sonicated (40 Hz, 5 s^{-1}) (Virsonic) and centrifuged (10,000×*g*, 10 min, 4 °C). The supernatant was used for the analysis. AchE activity was determined using Ellman et al. (1961) method by measuring the change in absorbance at 412 nm for 10 min. Activity values were normalized to the protein content, which was measured using Pierce 660 nm Protein Assay reagent (Thermo Fisher Scientific). The measurements were carried out in triplicates.

2.9. Integrated multi-level biomarker response

A general stress index, the IBRv2 developed by Sanchez et al. (2013) was calculated. All the biomarker responses investigated in this study were included in the calculation as well as those previously described in Schmitz et al. (2018) which were obtained during the same experiment, namely the plasma levels in vitellogenin (VTG), 17β-estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT).

The standardized value of each biomarker was estimated as follows:

$$Y_i = \log(\frac{X_i}{X_0})$$

Where X_i is the mean value of each individual biomarker and X_0 the mean value for the biomarker in the control group.

Y_i is then standardized with μ and σ being the general mean and the standard deviation of Y_i:

$$Z_{i=\frac{(Y_i-\mu)}{\sigma}}$$

The control group is considered here as a standard population. The biomarker responses in this group are centered to zero and a deviation index (*A*) is measured by subtracting the mean of the control group *Z0* to the mean of the treated groups *Zi*.

$$A = Z_i - Z_0$$

Finally, the function related IBR values were calculated as follows:

$$IBR = \sum(|A|/n)$$

With *n* being the number of biomarkers used to assess the impact of the mixture on each of the five different physiological functions studied. Finally, star plots were used to represent the scores obtained for each function. The calculation of IBR values and the star plots were performed using Excel software.

2.10. Statistical analysis

Biomarker responses are expressed as the mean ± standard error of

the mean (SEM). Analyses were performed using JMP, version 14 software (SAS Institute Inc., Cary, NC, 1989–2007) and figures were drawn with GraphPad Prism version 6.0 (Graphpad software, La Jolla CA, USA). Normality and homogeneity of variance were checked using Shapiro-Wilk and Bartlett's tests, respectively. When these criteria were not met, a logarithmic transformation was applied to the dataset. Data were analyzed using a two-way ANOVA considering the exposure time and the tested concentrations as fixed factors. Statistics were performed using the tanks as the experimental unit. Afterwards, to measure the effect of the tested concentrations at a specific time point, four within-day ANOVA were performed (day 1, 7, 21, and 42), followed by pairwise comparisons using Tukey's test. The level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Pharmaceutical exposure conditions and bioconcentration in fish tissues

Little is known about the complex interactions occurring between pharmaceuticals in mixtures and how they can influence both their bioavailability and toxicity. In order to identify these interactions and which compounds are responsible for the observed effects, it is recommended to assess the toxicity of each individual compound separately and then in mixture. However, this approach requires important logistic and equipment. Based on previous research, we assumed that at the tested concentrations each individual pharmaceutical would prove little impact on the studied biomarkers. In these conditions, investing the impact of pharmaceuticals in the form of a complex mixture seems to be a more accurate way to predict the consequences of the exposure conditions encountered in the environment (Mehinto et al., 2010).

The studied mixture intended to be representative of the conditions found in freshwater ecosystems. The selected compounds differ by their MOA and therefore biological targets. Indeed, analgesics and AINS MOA mostly relies on the COXs inhibition to prevent the production of pro-inflammatory prostaglandins (Praskova et al., 2012), while the neuroleptic carbamazepine acts on the nervous system and modulates the transduction of action potentials by inhibiting the activity of voltage-dependant sodium channel (Ambrósio et al., 2002). Finally, the antihypertensive drug irbersartan regulates the blood pressure by being a selective antagonist of the angiotensin II receptor (Bayer et al., 2014; Chapy et al., 2015).

In these conditions, an additive effect of the mixture through the independent action of each compound is often observed. The co-occurrence of different AINS in the mixture can also result in a concentration addition effect. Beside toxicity, interactions between pharmaceuticals can also affect their bioavailability. Indeed, antagonistic interactions resulting in a reduced bioavailability and biological activity have been observed in pharmaceutical mixtures similar to the one investigated here (Ding et al., 2016; Quinn et al., 2009).

Here, none of the compounds were detected in any of the trout sampled tissues for the different treatments and sampling times. As previously discussed in Schmitz et al. (2018), these results suggest a low bioconcentration of the tested pharmaceuticals due to their rapid metabolization and excretion. Potential antagonistic interactions occurring in the mixture could also provide an explanation for the absence of pharmaceuticals bioconcentration in fish tissues and the moderate impact of the mixture on fish health. Additionally, in the present experiment, we only investigated the effect of waterborne exposure on the mixture bioconcentration and toxicity. The effects of dietary exposure to pharmaceutical mixtures should also be taken into consideration, and especially when using model species located at a high trophic level such as the rainbow trout. Indeed, foodborne exposure can represent an important route of entrance for PPCPs which display high bioconcentration potential (Ebele et al., 2017; Vaclavik et al., 2020; Wang et al., 2021).

3.2. Survival and growth

All the fish remained healthy, and no mortality occurred during the experiment. The exposure did not affect fish growth. SGR values did not significantly differ between the control ($1.09 \pm 0.16\%$ day^{-1}) and the mixture-exposed fish (1.26 ± 0.2, 1.04 ± 0.38 and $1.14 \pm 0.36\%$ day^{-1} for the 1×, 10× and 100× conditions respectively).

3.3. Pharmaceutical mixture, immune system and oxidative stress status

Fish innate immune mechanisms are sensitive to stressors and can be specifically targeted by pharmaceuticals as they are highly conserved among vertebrates. They can therefore be used as early indicators of fish and aquatic environment health. Two key innate immune parameters involved in the inflammatory response, and acting against viral and bacterial infections, the plasma alternative complement activity (ACH50), and the lysozyme activity were assayed (Fig. 1) (Bols et al., 2001; Dautremepuits et al., 2006).

Exposure to the 100× concentration significantly decreased complement activity after 24 h, and induced it after 21 days ($p < 0.05$) (Fig. 1A). The cyclooxygenases COX-1 and COX-2. play key roles in the innate immune system by triggering the inflammatory response and their inhibition by analgesics and NSAIDs such as diclofenac can interfere with the complement cascade of chronically exposed fish (Ribas et al., 2016; Wang et al., 2016). Lysozyme activity was significantly higher in the 100× group ($p < 0.01$). The highest values were measured at 42 days, with the 100× mean activity being 1.4-fold higher than the control one ($p < 0.01$) (Fig. 1B). Pharmaceutical's impact on this biomarker in fish has been poorly investigated so far. Hoeger et al. (2005) did not observe any change in lysozyme activity in brown trout (*Salmo trutta* f. *fario*) exposed to diclofenac (0.5 μg L^{-1}, 21 days). In another aquatic model species, the green mussel (*Perna viridis*), exposure to carbamazepine (0.01 μg L^{-1}, 7 days) had inhibitory effects on lysozyme activity (Juhel et al., 2017). The stimulation of lysozyme activity is acknowledged as an indicator of general stress, which suggests that the experimental conditions affected the homeostasis of the organisms (Bols et al., 2001).

Oxidative stress is one of the most observed outcomes in fish following exposure to pharmaceuticals. Histological damages resulting from oxidative stress have been reported several times in organisms exposed to diclofenac, carbamazepine and paracetamol (Deng et al., 2006; Guiloski et al., 2017; Kumar et al., 2004; Li et al., 2010a). Liver tissues are especially affected as pharmaceuticals tend to accumulate preferentially in this organ where the detoxification process takes place.

In the present study, the hepatic detoxification mechanisms, EROD and GST were not affected by the mixture (Fig. 2A-B) regardless of the concentration and exposure time and remained at their constitutive low level during the entire experiment. These results are in contradiction with previous studies. Indeed, EROD activity is widely employed as an indicator of environmental pollution as it is known to be highly inducible by a large range of contaminants, including pharmaceuticals (Li et al., 2011). For instance, alterations in this biotransformation pathway have been highlighted in black catfish exposed to paracetamol (0.25 μg L^{-1}, 21 days), in rainbow trout exposed to high concentrations of carbamazepine (200 μg L^{-1}, 42 days), and in crucian carp (*Carassius carassius*) exposed to a mixture of diclofenac and carbamazepine (1:1, 2 μg L^{-1}, 7 days) (Guiloski et al., 2015, 2017; Li et al., 2010a; Nkoom et al., 2020).

Additionally to GST, which can be considered as a detoxification and antioxidant mechanism as it catalyzes the conjugation of the GSH cofactor on both xenobiotics and ROS to reduce their toxicity (Nunes et al., 2015), the activity of two antioxidant enzymes, CAT and GPx, as well as the concentrations in the GSH cofactor were measured.

Total GSH content varied according to the exposure time and treatment ($p < 0.05$) (Fig. 3C). Significantly reduced amounts of GSH were measured in all exposed fish after 24 h. The same trend was also

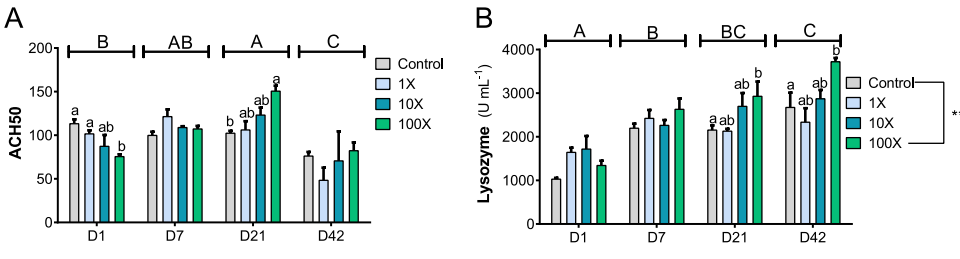

Fig. 1. Immunotoxicity biomarker responses in rainbow trout juveniles exposed to the pharmaceutical mixture. (A) Alternative complement pathway activity (ACH50), and (B) plasma lysozyme activity in the control, at the environmental concentration of the mixture (1×), 10 times (10×) and 100 times (100×) this concentration at different sampling times. Values represent the mean ± SEM (N = 4 tanks). Capital letters indicate significant differences between sampling times. Asterisks indicate significant differences between treatments. Small letters indicate significant differences between treatments at a certain time point.

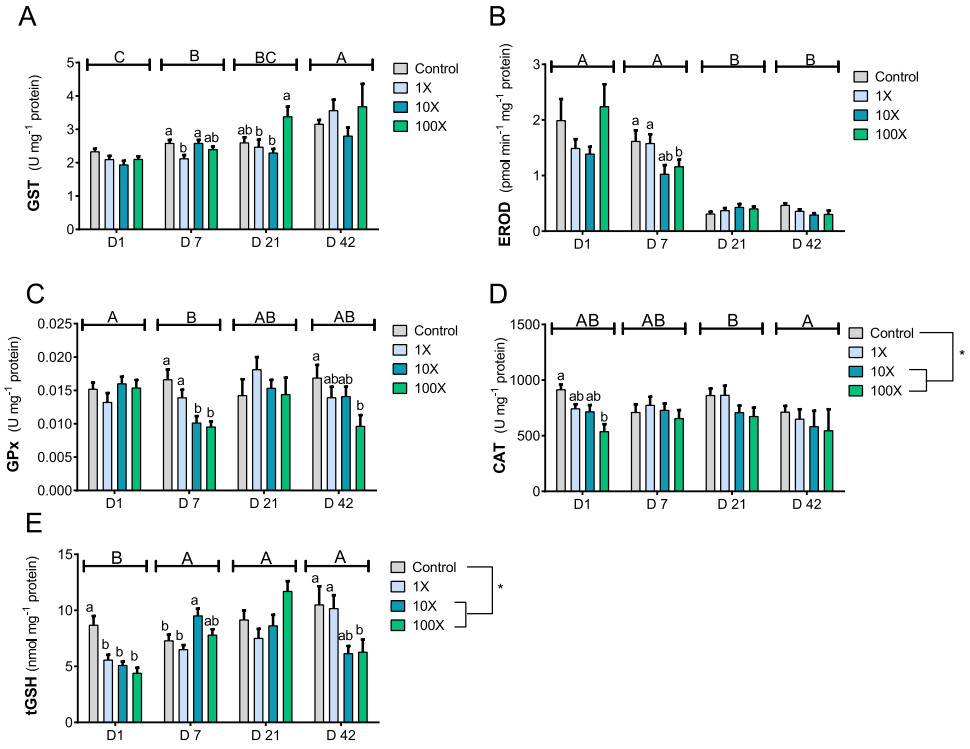

Fig. 2. Hepatic detoxification and oxidative stress biomarker responses in rainbow trout juveniles exposed to the pharmaceutical mixture. (A) GST, (B) EROD (C) GPx, (D) CAT, and (E) tGSH in the control and at the environmental concentration of the mixture (1×), 10 times (10×) and 100 times (100×) this concentration at different sampling times. Values represent the mean ± SEM (N = 4). Capital letters indicate significant differences between sampling times. Asterisks indicate significant differences between the treatments. Small letters indicate significant differences between treatments at a certain time point.

observed on day 42, but the difference was only significant between the control and the 10× group. GSH depletion has already been described in common carp (*Cyprinus carpio*) exposed to naproxen and was associated with oxidative stress (Sehonova et al., 2017). As GSH is also the cofactor of the GPx, the observed reduction of its content could explain the concentration-dependent decrease in GPx activity (Fig. 2D) measured after 7 and 42 days ($p < 0.05$). Regarding CAT activity (Fig. 2D), it varied according to the time ($p < 0.05$) and the treatment ($p < 0.05$). Significantly lower values were observed in the group 100× after 1 day ($p < 0.05$). This trend was maintained during the whole experiment but not in a statistically significant way. Both CAT and GPx activities were inhibited by carbamazepine exposure in zebrafish (*Danio rerio*) and rainbow trout (10 μg L^{-1}, 63 days and 200 μg L^{-1}, 42 days) and in tench (*Tinca tinca*) exposed to diclofenac (60 μg L^{-1}, 35 days) (da Silva Santos et al., 2018; Li et al., 2010b). Stancova et al. (2017, 2014) also reported an inhibitory effect of diclofenac on CAT activity in tench. However, when combined with carbamazepine and ibuprofen, diclofenac (1:1:1, 60 μg L^{-1}, 35 days) did not affect the activity of the enzyme, suggesting the occurrence of antagonistic interactions between these three compounds.

While previous studies highlighted important oxidative damages in fish exposed to the single pharmaceutical compounds, the oxidative stress caused by the present mixture in the liver tissues was minor. Hepatotoxic damages are usually related to the accumulation of toxic metabolites intermediates as NAPQI (N-acetyl-p-benzoquinone imine) for paracetamol (McGill and Jaeschke, 2013; Ramos et al., 2014; Stancova et al., 2017) or the formation of protein adducts in the case of diclofenac accumulation (Atchison et al., 2000). Our results suggest that the detoxification mechanisms seemed to be efficient enough to rapidly eliminate the assimilated compounds and therefore to prevent the development of oxidative damages and the activation of antioxidant mechanisms.

As previous studies were mostly investigating the effect caused by unrealistically high concentrations of contaminants, the comparatively low and environmentally relevant concentrations tested here could explain the observed differences between the present results and the ones reported in the literature. Another possible explanation is that, as suggested by Sehonova et al. (2017), the presence in the mixture of some compound classes as analgesics may have protective effects and reduce the oxidative stress caused by the other molecules.

3.4. Pharmaceutical mixture's neurotoxicity

The tested mixture showed little effects on the cholinergic system of the exposed fish. Except for a significant difference between the 1X group and the control at 7 days, no changes in AchE activity (Fig. 3A)

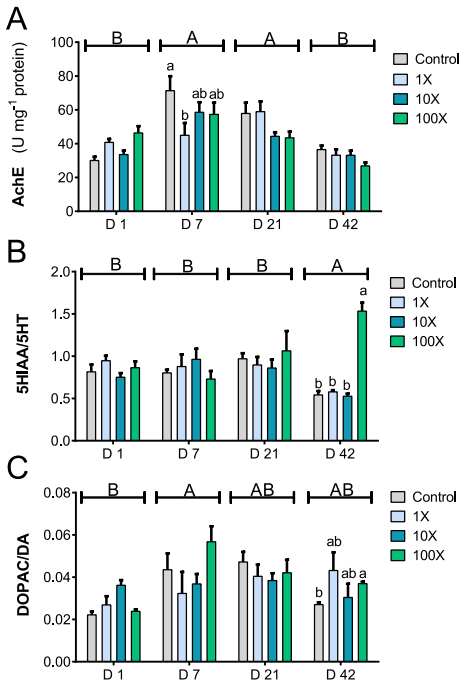

Fig. 3. Neurotoxicity biomarkers responses in rainbow trout juveniles exposed to the pharmaceutical mixture. (A) muscle AchE, (B) brain 5HIAA/5-HT ratio, and (C) brain DOPAC/DA ratio in the control, at the environmental concentration of the mixture (1×), 10 times (10×) and 100 times (100×) this concentration at different sampling times. Values represent the mean ± SEM (N = 4 tanks). Capital letters indicate significant differences between sampling times. Small letters indicate significant differences between treatments at a certain time point.

were measured. AchE is the target of numerous types of contaminants, and the inhibition of its activity is commonly used as an indicator of neurotoxicity (Fulton and Key, 2001). While pesticides as carbamates and organophosphates affect AchE activity directly by their binding to the protein, the studied pharmaceuticals tend to have indirect MOAs. AchE activity can for instance be modulated by the induction of an oxidative stress status as acetylcholine is the mediator of the cholinergic anti-inflammatory pathway (Rajendran et al., 2015). In zebrafish exposed to carbamazepine (10 μg L^{-1}, 63 days), mosquitofish (*Phalloceros harpagos*) exposed to paracetamol (80 μg L^{-1}, 96 h), and black catfish exposed to the NSAID ibuprofen (1 μg L^{-1}, 14 days) the induction of AchE activity was reported and correlated to the oxidative stress caused by these pharmaceuticals (da Silva Santos et al., 2018; Mathias et al., 2018; Pereira et al., 2018). Therefore, the absence of effects on the cholinergic system observed here is consistent with the minor oxidative stress response caused by the mixture.

Both brain serotoninergic (5HT/5HIA ratio) (Fig. 3B) and dopaminergic (DOPAC/DA ratio) (Fig. 3C) activities were significantly induced by the higher concentration of the mixture after 42 days ($p < 0.05$). However, as these effects were only reported at one sampling time, it is difficult to say if they can be considered as a punctual change or as the result of the exposure to the mixture.

Several compounds present in the mixture are known to have MOA targeting the nervous system. Carbamazepine acts mostly on voltage-dependent sodium channels, but it is also known to affect dopaminergic and serotoninergic activities by enhancing both DA and 5HT release and turnover (Ambrósio et al., 2002), and paracetamol MOA relies partly on the serotoninergic descending neuronal pathway stimulation (Jozwiak-Bebenista and Nowak, 2014).

Dopamine and 5HT play important roles in modulating gonadotropin production through the regulation of the hypothalamus-pituitary-gonad-liver (HPGL) axis (Trudeau et al., 2005). Guiloski et al. (2017) correlated the increase in brain 5HIAA/5HT ratio and DA concentrations to the reduction of the testosterone level in male black catfish exposed to paracetamol (2.5 μg L^{-1}, 21 days). The observed changes in DA and 5HT turnover may therefore be related to the endocrine disruptive effects observed during this study which have been described in Schmitz et al. (2018). Serotoninergic alterations could also result in immunosuppressive effects as the implication of 5-HT in the regulation of immune mechanisms has been demonstrated in both mammals (Herr et al., 2017) and fish (Duffy-Whritenour and Zelikoff, 2008; Ferriere et al., 1996).

3.5. IBR calculation

IBR index is mostly employed in field studies to discriminate contaminated from non-contaminated sites. However, it can also be used to highlight a response pattern representative of a specific type of contamination such as the pharmaceutical mixture studied here and to qualitatively assess the stress status of the exposed organisms (Arzate-Cárdenas and Martínez-Jerónimo, 2011; Maulvault et al., 2019; Perussolo et al., 2019; Trombini et al., 2019).

IBR results are represented as star plots in Fig. 4A. As highlighted by Broeg and Lehtonen (2006), the number of biomarkers included in the index increases its robustness but also reduces the relative weight of each biomarker. To increase the robustness of the present index, the responses of biomarkers representative of the reproductive system which were obtained during the same experiment and already described and discussed in Schmitz et al. (2018) were added to the ones studied here. To take into account the number of biomarkers and to simplify the interpretation of the results, the mean score was calculated for each studied physiological function. These scores indicate to which extend the biomarker responses of the exposed fish differ from those measured in the control group. These scores were then summed up to obtain the total IBR scores which are shown in Fig. 4B and can be used as general stress indicators. Total IBR scores decreased over time in 1× and 10× groups, suggesting a progressive reduction of the general stress induced by the mixture and a potential adaptation of fish to this stressor. Conversely, the time-dependent increase of 100× group IBR value suggests the development of a chronic stress response. Except for a time-dependent increase in the scores of the nervous and immune systems at the 100× concentration, the use of the IBR did not highlight any concentration or time-related pattern of response for the tested biomarkers. As underlined previously, small alterations in the functioning of the nervous system may have repercussions on other physiological systems, including the immune and the reproductive ones. Neurotoxicity biomarkers may therefore be employed as early indicators of the impact of pharmaceutical mixtures on aquatic organisms. Overall, these observations are in accordance with the results previously discussed for the individual biomarkers and support the fact that the waterborne exposure to the studied mixture poses a moderate risk for juvenile rainbow trout at its environmental concentration.

4. Conclusion

The present study shows that subchronic waterborne exposure to a mixture of commonly consumed pharmaceuticals at environmentally relevant concentrations had little effects on juvenile rainbow trout's key physiological functions. Immunomodulatory and potential neuroendocrine disruptive effects were observed but at an effective concentration 100 times superior to the median environmental one. The mixture's toxicity was lower than assumed based on previously reported toxic effects of the individual compounds contained in it. Our findings imply the importance of investigating mixture interactions as they can modulate drugs bioavailability and toxicity. We also enforce the statement that environmental risk assessment should not only rely on single compound toxicity measurements and should be carried out at environmentally realistic concentrations in order to avoid misestimating the actual risk posed by xenobiotics to aquatic organisms. To this end, the

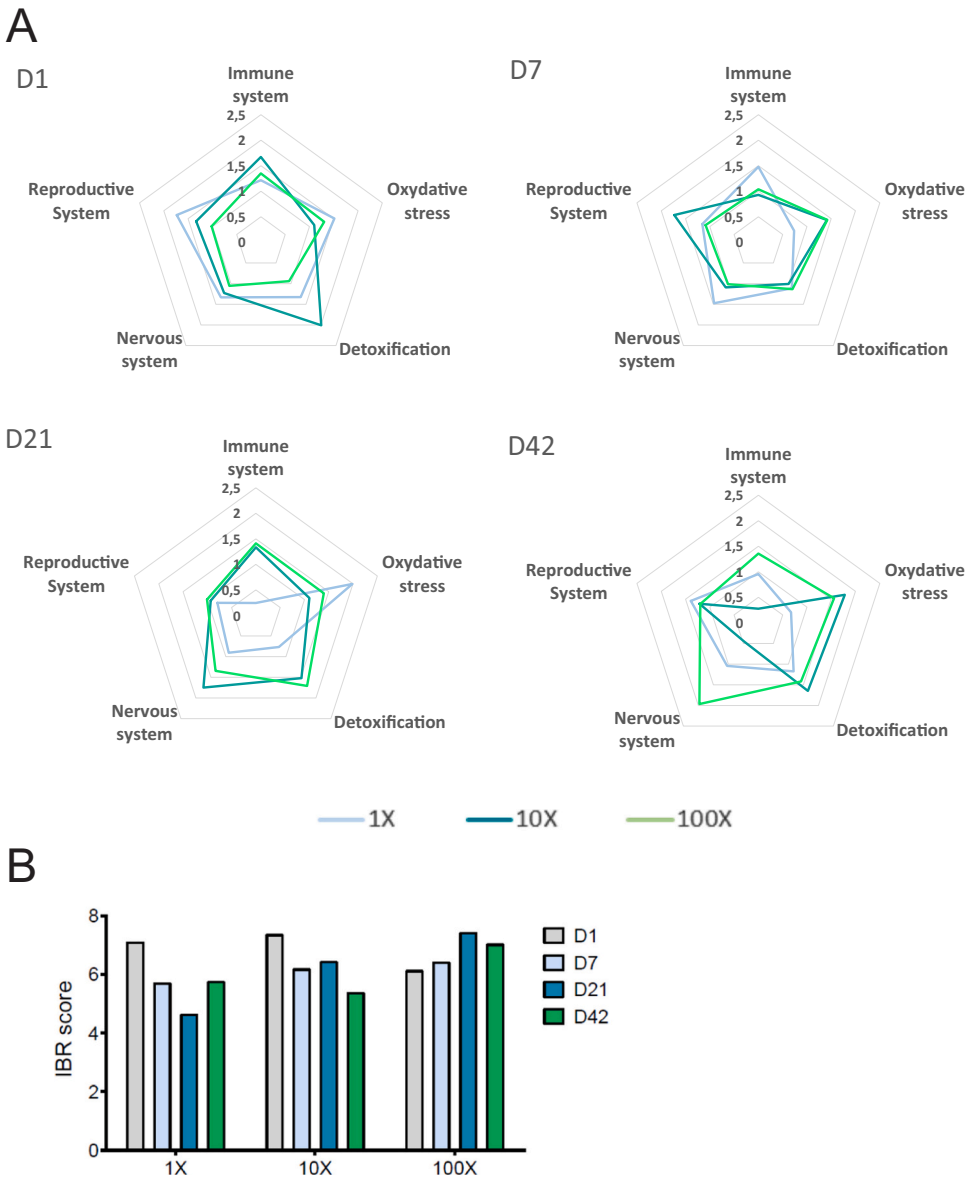

Fig. 4. (A) Integrated biomarker response of rainbow trout juvenile with the exposure to the three concentrations of the pharmaceutical mixture (1×, 10× and 100×) after 1, 7, 21, and 42 days of exposure. (B) Total IBR scores.

use of a holistic and integrative method encompassing all the physiological functions susceptible to be affected by the pollutants seems a promising approach.

CRediT authorship contribution statement

Mahaut Beghin: Methodology, Investigation, Data analysis, Writing - original draft, Writing - review & editing, Visualization. **Mélodie Schmitz:** Methodology, Investigation. **Stéphane Betoulle:** Investigation. **Olivier Palluel:** Investigation. **Sébastien Baekelandt:** Investigation. **Syaghalirwa N.M. Mandiki:** Investigation. **Erin Gillet:** Investigation. **Katherine Nott:** Investigation, Methodology. **Jean-Marc Porcher:** Conceptualization, Project administration. **Christelle Robert:** Investigation, Conceptualization. **Sébastien Ronkart:** Project administration. **Patrick Kestemont:** Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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