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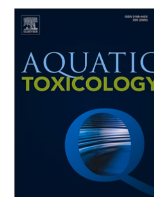

Novel organ-specific effects of Ketoprofen and its enantiomer, dexketoprofen on toxicological response transcripts and their functional products in salmon

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ABSTRACT

Racemic ketoprofen (RS-KP) and its enantiomer, dexketoprofen (S(+)-KP) are widely used non-steroidal anti-inflammatory drugs (NSAIDs), and commonly detected in the aquatic environment. The present study has evaluated the toxicological effects of RS-KP and S(+)-KP on biotransformation and oxidative stress responses in gills and liver of Atlantic salmon. Fish were exposed for 10 days using different concentrations of RS-KP (1, 10 and 100 μg/L) and S(+)-KP (0.5, 5 and 50 μg/L). Biotransformation and oxidative stress responses were analysed at both transcript and functional levels. In the gills, significant inhibitory effect at transcriptional and enzymatic levels were observed for biotransformation and oxidative stress responses. On the contrary, biotransformation responses were significantly increased at transcriptional and translational levels in the liver, while the associated enzymatic activities did not parallel this trend and were inhibited and further demonstrated by principal component analysis (PCA). Our findings showed that both compounds produced comparable toxicological effects, by producing organ-specific effect differences. RS-KP and S(+)-KP did not bioaccumulate in fish muscle, either due to rapid metabolism or excretion as a result of their hydrophobic properties. Interestingly, the inhibitory effects observed in the gills suggest that these drugs may not undergo first pass metabolism, that might result to downstream differences in toxicological outcomes.

1. Introduction

Pharmaceuticals are a big class of emerging environmental pollutants widely used daily for different purposes. The concern about these compounds is due to their accelerating usage and perpetual disposal resulting in chronic-persistent presence in the environment and potential toxicity towards non-target organisms (aus der Beek et al., 2016; Yin et al., 2017). Pharmaceuticals end up in different environmental compartments through multiple routes, and over the past two decades an increasing number of reports have measured traces of various prescription and over-the-counter pharmaceuticals (Jones et al., 2001; Herberer, 2002; Fent et al., 2006; Gworek et al., 2019). Pharmaceuticals mainly enter into environmental compartment as a consequence of intense private usage and are released either as parent compound or as metabolized and transformed molecules (Williams, 2005). Furthermore, the quantity of pharmaceutical products that are discharged into the environment exceeds the transformation capability in the environment (Nikolaou et al., 2007). Analysis of diverse aquatic environments led to the identification of more than 600 pharmaceuticals and personal care products (PPCPs), and transformed products in surface water, groundwater, sewage treatment plants (STP), wastewater treatment plants (WWTP) and soil (IWW, 2014; Kümmerer, 2013). Once excreted, pharmaceuticals may have different fate – they may undergo biodegradation, either remain suspended or dissolved in water, bind to biosolids or sewage sludge (Trudeau et al., 2005). The degradation rate of pharmaceuticals depends on their chemical proprieties and environmental compartment they reach (Fent et al., 2006).

Non-steroidal anti-inflammatory drugs (NSAIDs) are considered one

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of the most used group of pharmaceuticals worldwide due to their availability, low cost and absence of addictive side effects (Asghar et al., 2015). NSAIDs are regularly detected in both WWTP and surface waters at low concentrations, although the continuous and chronic exposure to low concentrations may produce toxic effects (Fent et al., 2006; Weigel et al., 2004; Kafaei et al., 2018). Among NSAIDs, Ketoprofen (KP) is one of the most used and frequently detected compounds in many countries. A large number of studies have reported the detection of KP in different environmental compartments at concentrations, ranging from 250 ng/L to 5.7 μg/L (Fent et al., 2006; Murray et al., 2010; Spongberg et al., 2011; Lapworth et al., 2012; Marsik et al., 2017). KP is a racemic mixture characterized by an equal amount of two enantiomers, R(-)-KP and S(+)-KP (Fent et al., 2006). However, the anti-inflammatory effect of the racemic RS—KP, resides almost exclusively in the S(+)-enantiomer (Barbanoj et al., 2001). Consequently, the efficacy of the S(+)-enantiomer has generated high interest in the commercialization of the dextrorotatory enantiomer (dexketoprofen: S(+)-KP) (Agranat et al., 2002). The enantiomeric distribution of NSAIDs in the aquatic environment depends on the extent of both the chiral inversion of R-enantiomers during human metabolism and preferential biodegradation of S-enantiomer during wastewater treatment (Caballo et al., 2015). Recently, ecotoxicological effects of both the racemic and S(+)-KP on non-target organisms (bacteria, algae, crustaceans) were reported (Mennillo et al., 2018a, b). In these studies, we also evaluated the toxicity of these compounds by investigating the biotransformation (cytochrome P450) and oxidative stress pathways in two different *in vitro* cell models (Mennillo et al., 2018a, b).

The cytochrome P450 (CYP)-dependent monooxygenase system plays a pivot role in the oxidative metabolism or biotransformation of a wide range of foreign compounds, including drugs (Anzenbacher and Anzenbacherová, 2001; Kulcsár et al., 2016). Induction of Cyp1a at transcriptional, translational and catalytic levels in fish organs such as the liver and gills, is routinely used as biomarker of exposure to environmental contaminants (Oris and Roberts, 2009; Jönsson et al., 2006). Specifically in the gills, CYP-catalysed first-pass metabolism may take place, resulting to decreases in systemic bioavailability of rapidly metabolized waterborne pollutants Jönsson et al., 2006; Andersson and Pärt, 1989; Levine and Oris, 1999). A high number of pharmaceuticals are metabolized by CYP enzyme in fish, thus CYP enzymes are of critical importance for both detoxification and formation of toxic metabolites (Dorne et al., 2007). Moreover, there are evidences showing that pharmaceutical products may also activate oxidative stress by increasing reactive oxygen species (ROS) production and subsequently modulating antioxidant responses (Mennillo et al., 2018b(Dorne et al., 2007; Osburn and Kensler, 2008). The antioxidant system plays an important role in the maintenance of cellular homeostasis in organisms (ibid).

Based on our knowledge, studies on toxicological responses related to biotransformation and oxidative stress, after exposure to either RS-KP or S(+)-KP are very limited or almost non-existent in fish. Therefore, the aim of the present study is to evaluate the *in vivo* differential organ- and compound-specific toxicological effects of these pharmaceuticals using fish model and at both transcriptional and functional product levels. Biotransformation and antioxidant pathways were examined in the liver and gills of salmon exposed to different waterborne concentrations of RS-KP and S(+)-KP. Our hypothesis is that waterborne exposure of salmon to RS-KP and S(+)-KP will produce organ- and compound-specific effects on anti-oxidant and biotransformation responses at transcriptional and functional products (enzyme and proteins) levels, and indicative of toxicological response patterns in the exposed animals.

2. Materials and methods

2.1. Chemicals and reagents

Pharmaceuticals ketoprofen RS-KP (CAS: 22071−15-4) and dexketoprofen S(+)-KP (CAS: 22161−81-5) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical standards with purity of 99 %. Stock standard solutions of pharmaceuticals were prepared the same day of exposure in deionized water at 10 mg/L. Amber glassware was used to prevent light degradation of pharmaceuticals.

2.2. Experimental animals

Atlantic salmon (*Salmo salar*) juveniles were obtained from Settefiskanlegget Lundamo AS (Lundamo, Norway) and brought to the animal holding facilities of the Norwegian University of Science and Technology (NTNU), Department of Biology (Sealab, NTNU), where acclimation and exposure was performed. Fish were acclimated for a period of 9 days, in a 100-L tank with continuously running freshwater at 10 °C, using a 12:12 h photoperiod. The animals were given food once during acclimation, and thereafter starved during the exposure period, to avoid possible interaction with contaminant absorption processes and antioxidants in fish feed.

2.3. Exposure and sampling

The fish were divided into six exposure groups and two control groups, of 6 individuals each. The treatment groups were exposed for 10 days to three different nominal concentrations for each compound (RS-KP: 1, 10 and 100 μg/L or S(+)-KP: 0.5, 5 and 50 μg/L). The low (RS-KP: 1 and S(+)-KP: 0.5) and medium (RS-KP: 10 and S(+)-KP: 5) concentrations were chosen to resemble the environmental relevant concentrations, while the high concentration (RS-KP: 100 and S(+)-KP: 50) were used to mimic an extreme condition. Each group was kept in a separate 70 L glass aquarium with continuously aerated freshwater. The water (80 %) was changed every five days, and new treatment solution was added to restore the desired concentration within each tank. See SI for detailed sampling protocol.

2.4. Quantitative (real-time) PCR

Total RNA was isolated directly from the liver and gills samples performed using the Direct-zol RNA MiniPrep RNA isolation kit, following the manufacturer's instructions. RNA samples integrity was confirmed by spectrophotometric analysis and formaldehyde agarose gel electrophoresis. cDNA was generated using the iScript cDNA synthesis kit, as described by the supplier (Bio-Rad). Real-time PCRs were performed with gene-specific primers (SI Table S3) using the Mx3000 P real-time PCR system (Stratagene, La Jolla, CA). Detailed real-time protocol is presented in the SI.

2.5. Post-mitochondrial supernatant (PMS) and biochemical assays

PMS fractions were prepared by centrifugation as described previously (Pesonen and Andersson, 1987) and presented in the SI.

The 7-ethoxyresirufin O-deethylase (EROD) and 7-methoxyresorufin O-demethylase (MROD) activities in the liver and gills were measured in PMS fractions from all exposure groups as described by (Burke and Mayer (1974)). Detailed protocol is presented in the SI.

Antioxidant enzyme activities were measured in gills and liver PMS fractions from the RS-KP (100 μg/L) and S(+)-KP (50 μg/L), only, due to limited sample materials. Glutathione peroxidase (Gpx), glutathione reductase (Gr), glutathione-s-transferase (Gst), catalase (Cat) activities were performed according to standard protocols and detailed protocols are presented in the SI.

Total amount of protein was determined with the method of (Bradford (1976)), using bovine serum albumin (BSA) as standard. All enzymes and protein measurements were performed using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA) for absorbance and fluorescence readings.

2.6. Immunoblotting assays

Total liver proteins (30 μg) was separated by 12 % precast sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE: Bio-Rad). Protein bands intensity were quantified using ImageJ software 1.52 t. Detailed immunoblotting protocol is presented in the SI.

2.7. Analytical chemistry

Concentrations of RS-KP and S(+)-KP were measured in experimental water solutions and fish muscle. Enantiomers were measured *via* an enantioselective high-pressure liquid chromatography- ultraviolet (HPLC-UV) detection method. Water samples were filtered and extracted with SPE (Strata™-XL 100 μm Polymeric Reversed Phase) followed by HPLC analysis with a chromatographic system that consisted of a Series 200 PerkinElmer gradient Pump coupled to a Series 200 PerkinElmer variable UV detector, which was set at 254 nm. Detailed protocol is presented in the SI.

2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software Inc. 2012). One-way ANOVA followed by Dunnett's multiple comparison test was performed to evaluate differences between treatment and control groups in each organ. Two-way ANOVA followed by Sidak's multiple comparison test was performed to evaluate differences within exposure condition between liver and gills and to compare the effects of racemate and its enantiomer in each tissue. The level of statistical significance was set at $p < 0.05$. Principal component analysis (PCA) was applied to evaluate the relationship between RS—KP and S(+)-KP and biomarkers in liver and gills of control and exposure groups along the experimental period. This analysis was performed using the PAST 3.25 software.

3. Results

3.1. RS-KP and S(+)-KP concentrations in test solutions and fish

Concentrations of RS-KP and S(+)-KP in the experimental water was measured at day 0 and before renewal at day 5. The nominal exposure concentrations for R(-)KP and S(+)-KP in RS-KP solutions are reported in Table 1. At the nominal concentration of 100 μg/L of RS-KP, a similar reduction level was measured for both enantiomers in the presence or absence of fish. On the other hand, slight reduction values were observed at day 5, for the S(+)-KP nominal exposure concentrations of 0.5, 5 and 50 μg/L, with deviation percentages at: -14.1, 2.8, -8.1 and -10.9, respectively (Table 2). However, it is possible that these changes might be due to experimental error or adsorption to the walls of exposure tanks (Maharajan et al., 2018). The concentrations of RS-KP and S (+)-KP were also measured in fish muscles and were found to be below the detection limit (Data not shown).

Table 1
Nominal and measured concentrations of RS-KP [R(-) and S(+) enantiomer] in the different exposure tanks.

	*Measured concentration (μg/L)					
	Day 0		Day 5		Deviation (%) #	
Nominal concentration (μg/L)	R (-)	S (+)	R (-)	S (+)	R (-)	S(+)
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1	1.6 ± 0.1	1.2 ± 0.1	1.5 ± 0.2	2.0 ± 0.2	−9.3	67.2
10	4.1 ± 0.3	4.0 ± 0.2	5.8 ± 0.5	5.8 ± 0.6	39.8	43.8
100	64.0 ± 3.9	71.0 ± 5.3	55.0 ± 4.1	60.0 ± 5.0	−14.1	−15.5
100 without fish	64.0 ± 3.9	71.0 ± 5.3	60.0 ± 3.6	61.0 ± 3.4	−6.3	−14.1

n.d. = not detected. 0 = Control.
[*] Sum of 50 % R(-) and S(+) enantiomers.
[#] Deviation (%) = (Measured concentration (day 0 – day 5)/measured concentration (day 0) x 100).

3.2. Effects on biotransformation responses

Biotransformation responses were assessed in the liver and gills of the experimental animals. Fish exposed to RS—KP showed significant concentration-dependent increase (liver) or decrease (gills) for *cyp1a* mRNA levels (Fig. 1A). These exposure response pattern represent an opposing trend in the two organs after exposure (Fig. 1A). Specifically, the expression of *cyp1a* in gills was significantly decreased at 10 and 100 μg/L RS—KP, compared to the control group, while the expression of *cyp1a* in liver was significantly increased at the same concentrations of RS—KP (Fig. 1A).

On the functional product level, we observed that the gills and liver EROD activities were decreased after exposure to RS—KP, and significantly so in the gills at 10 and 100 μg/L RS—KP, compared to the control (Fig. 1C). Cyp1a protein levels measured by immunoblotting method showed concentration-dependent increase in protein band intensity in the liver samples (Fig. 3A).

The expression of gills *cyp1a* mRNA was significantly reduced after exposure to S(+)-KP at 5 and 50 μg/L, compared to controls (Fig. 1B). In the liver, we observed concentration-specific significant increase of *cyp1a* expression after exposure to S(+)-KP at 5 and 50 μg/L, compared to controls (Fig. 1B). For the EROD activity, we observed significant decrease after exposure to S(+)-KP at all tested concentration for the gills and liver (Fig. 1D). No significant changes in immunochemically measured Cyp1a protein was observed in liver samples after exposure to S(+)-KP (Fig. 3B).

For *cyp3a* transcript expression in the gills and liver, we observed similar exposure effects between RS-KP and S(+)-KP. The expression levels of *cyp3a* mRNA in gills significantly decreased below control, after exposure to RS-KP at 1 and 100 μg/L concentrations (Fig. 2A). In contrast, liver *cyp3a* transcript expression was significantly increased after exposure to RS-KP at 10 and 100 μg/L of (Fig. 2A). The effects of RS-KP paralleled the significant reduction (gills) and elevation (liver) of MROD activity at all exposure concentrations (Fig. 2C) and immunochemically determined Cyp3a protein expression levels in the liver (Fig. 3 C).

For the (S(+)-KP) enantiomer, gills *cyp3a* transcript levels were significantly decreased in fish at all exposure concentrations (Fig. 2B). In the liver, significant concentration-specific increase of *cyp3a* transcripts was observed after exposure to S(+)-KP (Fig. 2B). On the functional activity level, gills MROD decreased in a concentration-dependent manner after exposure to S(+)-KP (Fig. 2D). For the liver, MROD activity was significantly induced after exposure to 0.5 μg S(+)-KP/L, and

Table 2
Nominal and measured concentrations of S(+)-KP in the different exposure tanks.

	Measured concentration (μg/L)		
Nominal concentration (μg/L)	Day 0	Day 5	Deviation# (%)
0	n.d.	n.d.	n.d.
0.5	0.7 ± 0.1	0.7 ± 0.1	−14.1
5	7.5 ± 1.1	5.2 ± 1.5	2.8
50	63.3 ± 5.4	58.2 ± 4.9	−8.1
50 without fish	63.3 ± 5.4	56.4 ± 4	−10.9

n.d. = not detected. 0 = Control.
[#] Deviation (%) = (Measured concentration (day 0 – day 5)/measured concentration (day 0) x 100).

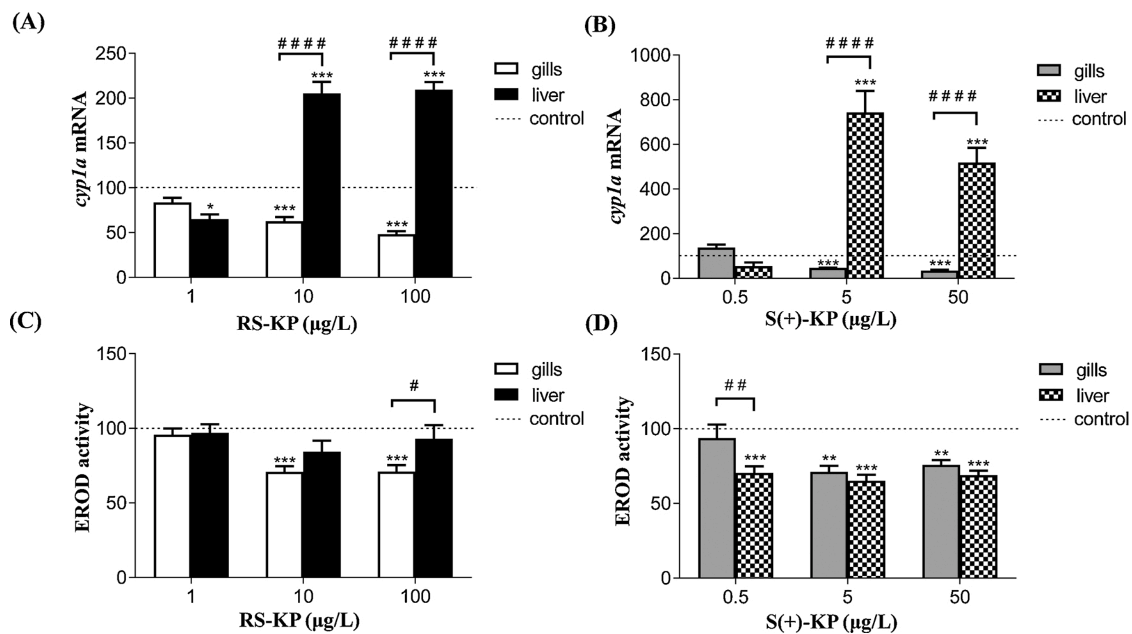

Fig. 1. Transcriptional changes of *cyp1a* in gills and liver (A and B), and EROD activity in gills and liver (C and D) of juvenile of Atlantic salmon after 10 days of exposure to RS—KP and S(+)-KP. Transcripts and enzymatic data are reported as mean ± standard error of the mean (SEM) and presented as percent (%) of control (n = 6). Significant differences between control and exposure groups in each organ are reported as *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001 (One-way ANOVA followed by Dunnett's multiple comparison test) and between organs at the same concentration are marked with $^{\#}$p ≤ 0.05, $^{\#\#}$p ≤ 0.01, $^{\#\#\#}$p ≤ 0.001 and $^{\#\#\#\#}$p ≤ 0.0001 (Two-way ANOVA followed by Sidak's multiple comparison test).

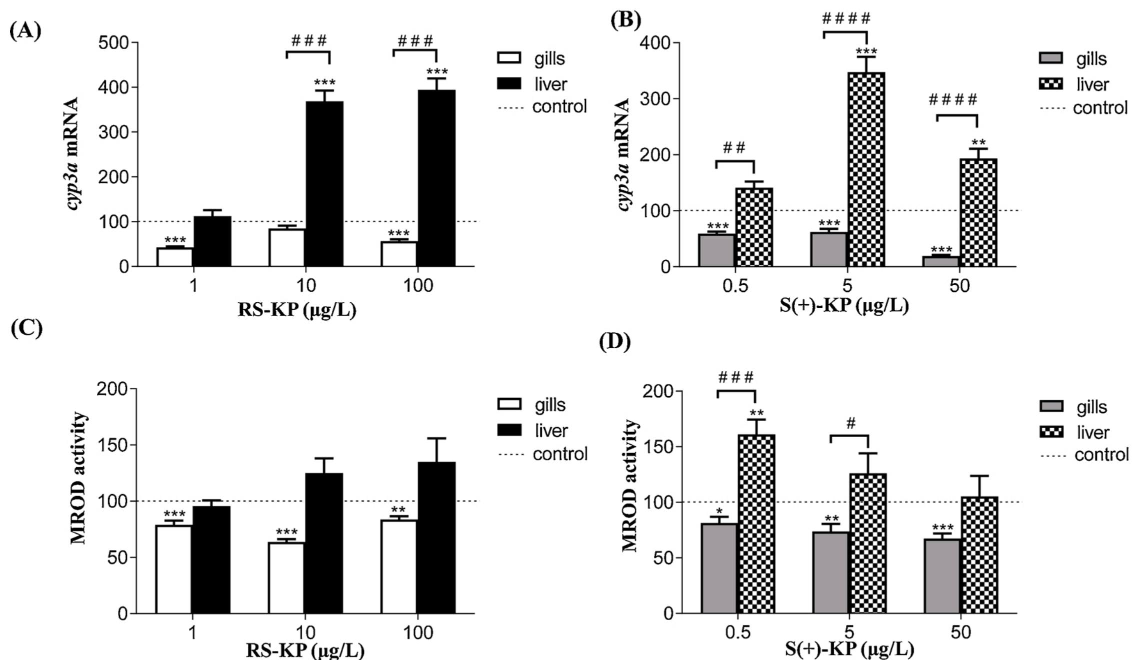

Fig. 2. Transcriptional changes of *cyp3a* in gills and liver (A and D), and MROD activity in gills and liver (C and D) of juvenile of Atlantic salmon after 10 days of exposure to RS—KP and S(+)-KP. Transcripts and enzymatic data are reported as mean ± standard error of the mean (SEM) and presented as percent (%) of control (n = 6). Significant differences between control and exposure groups in each organ are reported as *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001 (One-way ANOVA followed by Dunnett's multiple comparison test) and between organs at the same concentration are marked with $^{\#}$p ≤ 0.05, $^{\#\#}$p ≤ 0.01, $^{\#\#\#}$p ≤ 0.001 and $^{\#\#\#\#}$p ≤ 0.0001 (Two-way ANOVA followed by Sidak's multiple comparison test).

thereafter, a non-significant concentration-dependent decrease of liver MROD was observed (albeit above control levels: Fig. 2D). In general, the liver *cyp3a* transcript expression pattern paralleled increases in immunoblotted Cyp3a protein after exposure to S(+)-KP (Fig. 3D).

Although the biotransformation results showed similar responses for the racemate and its enantiomer, differences between RS-KP and S(+)-KP were evaluated for each concentration (Table 3). In particular, S(+)-KP exposure produced higher induction of liver *cyp1a* mRNA, compared to RS-KP at medium and high concentrations and stronger inhibition of EROD activity at the same exposure conditions. In addition, the expression of gill *cyp3a* mRNA was significantly reduced after exposed to S(+)-KP, compared to RS-KP, particularly at the medium-

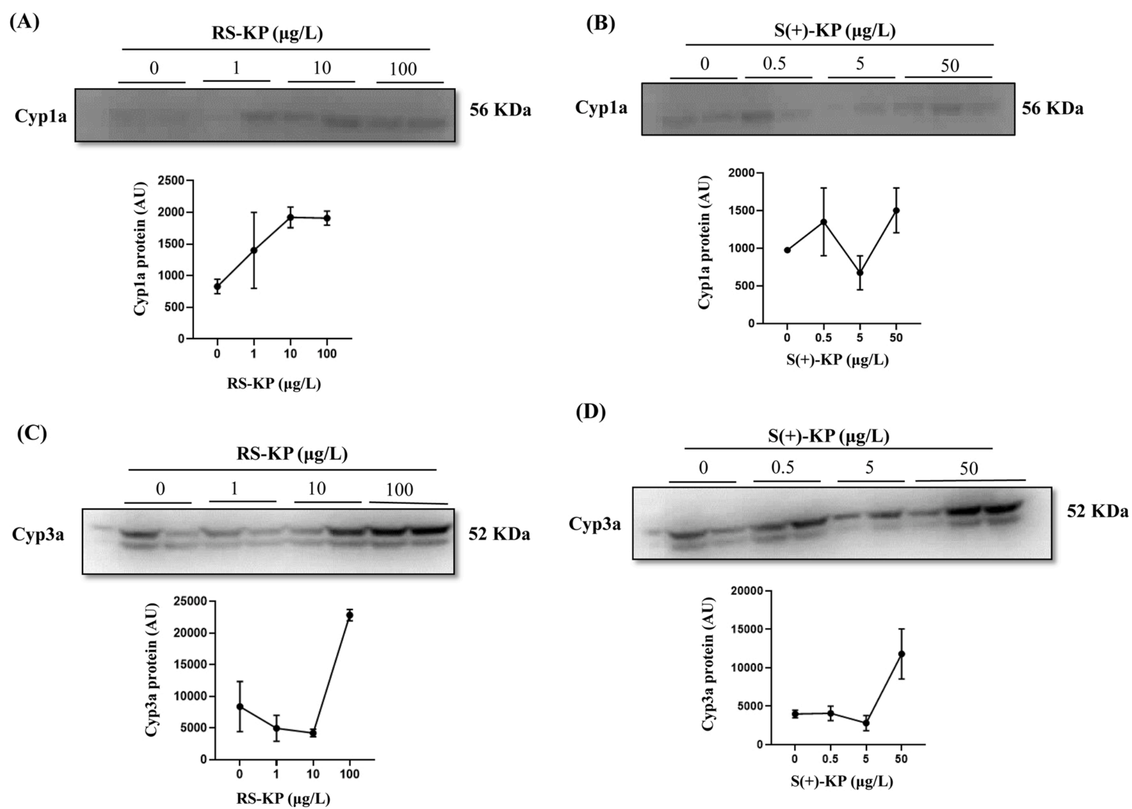

Fig. 3. Immunoblotting analysis using western blotting of Cyp1a (A and B) and Cyp3a (C and D) in the liver of juvenile of Atlantic salmon after 10 days of exposure to different concentrations of RS—KP and S(+)-KP. 30 µg of total protein concentration was loaded per well. Graphs below blotting images show scanned protein band intensity and all arbitrary unit (AU) values represent mean ± standard error of the mean (SEM; n = 2-3 protein bands).

and high exposure concentrations (Table 3).

3.3. Effects on antioxidant responses

Further assessment of toxicity was conducted by monitoring the levels of the principal oxidative stress markers (*gpx, gr, cat* and *gst*) in the gills and liver upon exposure to RS-KP and S(+)-KP. Transcriptional analysis of *gpx, gr, gst* and *cat* revealed concentration-specific significant decreases after exposure to RS-KP, compared to control (Fig. 4A, B, C and D, respectively). On the other hand, liver *gpx* and *gr* mRNA expression showed concentration-dependent significant increase after exposure to RS-KP (Fig. 4A and B). However, liver *gst* and *gr* expressions were significantly below control levels, after exposure to 1 µg RS-KP (Fig. 4A and B). Furthermore, liver *gst* and *cat* mRNA also showed RS-KP exposure-specific expression patterns, significantly increasing at 100 µg/L (*gst*) and at 10 and 100 µg/L (*cat*), compared to control (Fig. 4C and D, respectively). Gills *gst* and *cat* mRNA showed RS-KP exposure-specific expression pattern, that were generally below control levels (Fig. 4C and D, respectively).

Fish exposure to S(+)-KP produced significant concentration-dependent elevation in liver *gpx, gr, gst* and *cat* transcript levels, compared to control (Fig. 5A, B, C and D, respectively). For the gills, S(+)-KP produced significant concentration-dependent reduction (almost to total inhibition in some variables) in *gpx, gr, gst* and *cat* transcript levels, compared to control (Fig. 5A, B, C and D, respectively).

To further confirm the physiological relevance of the changes in transcription patterns, we measured Gpx, Gr, Gst and Cat enzymatic activities in the gills and liver of fish exposed to the highest (100 µg/L) concentrations of RS—KP and S(+)-KP (Fig. 6). In the gills, the enzymatic activities paralleled the transcript expression patterns, showing significant inhibition of all activities, compared to the control fish (Fig. 6A). On the hand, liver Gpx, Gr, Gst and Cat activities did not show significant changes, compared with control group and these were in contrast with the transcript expression patterns observed at the 100 µg/L exposure group (Fig. 6B).

The comparison between RS-KP and S(+)-KP showed that most of the antioxidant genes were induced higher in liver (*gpx, gr* and *gst*) or inhibited more in the gills, when exposed to S(+)-KP (Table 3).

3.4. Principal component analysis (PCA)

The relationship between all analytical observations and different exposure groups of the tested pharmaceuticals in the liver and gills is shown in Fig. 7. Principal component 1 and 2 (PC1 and PC2) together accounted for 87 % of the total variance. In particular, PC1 accounted for 70.4 %, showing the liver samples at the positive side of the axis, while gills samples were at the negative side. Considering the liver samples, both RS—KP concentrations (10 and 100 µg/L) exhibited high correlation with all molecular biomarker, while antioxidant response and biotransformation biomarkers displayed major correlation with S(+)-KP (5 and 50 µg/L). Coloured convex hulls defined each set of point for liver and gills exposed to RS—KP and S(+)-KP (Fig. 7) and overall PCA data is presented in SI Table S4.

4. Discussion

In the present study, the toxicity of RS-KP and S(+)-KP was evaluated by exposing salmon to different concentrations of these NSAIDs for a period of 10 days. Limited amount of studies on the toxicity of RS-KP are available, and non-existent for S(+)-KP. Previous reports have shown that low concentrations of RS-KP may produce significant changes at transcriptional levels, with strong impact on the representative trophic levels of the aquatic ecosystem (Illés et al., 2014; Mezzelani et al., 2016). In particular, we recently showed that biotransformation and oxidative

Table 3
Comparison of the analysed biomarker responses between RS-KP and S(+)KP in liver and gills at low (1 or 0.5 μg/L), medium (10 or 5 μg/L) and high (100 or 50 μg/L) concentrations. Data are presented as percentage (%) of control and reported as mean ± standard error of the mean (SEM). Significant differences between RS-KP and S(+)-KP are determined by Two-way ANOVA followed by Sidak's multiple comparison test. #Data are given as mean ± SEM.

Tissue	Exposure	Biomarker	RS-KP#	S(+)-KP#	Significant
Liver	Low	*cyp1a*	64.9 ± 5.4	54.7 ± 6.8	no
	Medium		**205.3 ± 12.9**	**743.3 ± 39.3**	**yes**
	High		**209.5 ± 8.4**	**519.2 ± 26.9**	**yes**
Gills	Low		**85.6 ± 5.2**	**138.0 ± 12.8**	**yes**
	Medium		62.6 ± 4.7	46.7 ± 1.3	no
	High		48.3 ± 3.3	34.9 ± 4.0	no
Liver	Low	EROD	**96.9 ± 5.7**	**70.4 ± 4.3**	**yes**
	Medium		84.4 ± 7.3	65.2 ± 4.1	no
	High		**92.9 ± 9.1**	**68.9 ± 3.0**	**yes**
Gills	Low		95.7 ± 4.1	75.8 ± 8.9	no
	Medium		70.8 ± 3.7	71.2 ± 4.0	no
	High		70.9 ± 4.3	75.8 ± 3.3	no
Liver	Low	*cyp3a*	112.1 ± 13.7	141.2 ± 11.1	no
	Medium		368.6 ± 24.3	347.6 ± 27.4	no
	High		**394.6 ± 24.3**	**193.5 ± 17.3**	**yes**
Gills	Low		42.6 ± 2.3	59.3 ± 3.6	no
	Medium		**84.6 ± 6.6**	**62.1 ± 5.8**	**yes**
	High		**56.5 ± 4.3**	**19.1 ± 2.0**	**yes**
Liver	Low	MROD	**95.5 ± 5.3**	**161.0 ± 13.4**	**yes**
	Medium		125.0 ± 13.1	126.1 ± 18.0	no
	High		134.9 ± 20.9	105.2 ± 18.5	no
Gills	Low		79.1 ± 3.7	81.5 ± 5.5	no
	Medium		63.8 ± 2.4	73.7 ± 6.9	no
	High		83.7 ± 2.8	67.4 ± 4.4	no
Liver	Low	*gpx*	83.3 ± 3.7	152.4 ± 12.7	no
	Medium		**177.5 ± 29.2**	**424.9 ± 51.6**	**yes**
	High		292.2 ± 33.7	399.2 ± 36.4	no
Gills	Low		13.1 ± 1.4	18.0 ± 4.3	no
	Medium		**85.9 ± 4.5**	**38.8 ± 14.2**	**yes**
	High		**47.3 ± 7.0**	**10.2 ± 2.9**	**yes**
Liver	Low	*gr*	**75.2 ± 8.9**	**220.9 ± 24.9**	**yes**
	Medium		**141.3 ± 2.2**	**320.9 ± 42.6**	**yes**
	High		**154.6 ± 13.3**	**420.6 ± 27.8**	**yes**
Gills	Low		19.6 ± 2.8	29.2 ± 4.1	no
	Medium		74.3 ± 6.5	69.6 ± 8.6	no
	High		**53.9 ± 6.8**	**12.1 ± 4.0**	**yes**
Liver	Low	*gst*	**30.7 ± 2.9**	**136.9 ± 31.4**	**yes**
	Medium		**91.5 ± 5.2**	**186.9 ± 24.1**	**yes**
	High		**124.0 ± 13.4**	**211.6 ± 29.8**	**yes**
Gills	Low		34.2 ± 6.1	35.2 ± 4.1	no
	Medium		**76.7 ± 2.7**	**46.4 ± 6.4**	**yes**
	High		**65.1 ± 8.6**	**18.4 ± 2.9**	**yes**
Liver	Low	*cat*	66.4 ± 5.8	90.4 ± 15.3	no
	Medium		172.9 ± 19.4	178.6 ± 22.4	no
	High		208.9 ± 15.1	185.9 ± 9.8	no
Gills	Low		**75.0 ± 7.2**	**47.0 ± 2.7**	**yes**
	Medium		74.5 ± 3.5	58.9 ± 3.8	no
	High		**64.3 ± 5.9**	**43.1 ± 7.6**	**yes**

stress pathways were activated and differentially modulated in cell lines exposed to these drugs (Mennillo et al., 2018a, b). Additionally, bioassays using non-target organisms such as algae and crustacean were severely impacted after chronic exposure to RS-KP and S(+)-KP (Mennillo et al., 2018a). In the present study, exposure to these drugs produced significant, but differing effects on the liver and gills, demonstrating that environmentally relevant concentrations of RS-KP and S(+)-KP activated toxicological and protective cellular responses in fish.

4.1. Biotransformation responses

The liver is the main metabolizing organ involved in detoxification and biotransformation of xenobiotics (Nunes et al., 2015). The relevance of using CYPs as biomarkers for toxicity has been documented over the years (Hong and Yang, 1997; Kroon et al., 2017). Among the CYPs, Cyp1a and Cyp3a represent the main enzymes involved in phase I biotransformation (Popovic et al., 2015). Our study showed that both compounds activated the AhR to produce *cyp1a* transcription. Despite the increase of hepatic *cyp1a* mRNA observed after 10 days of exposure, EROD activity in the liver was at the control levels in fish exposed to RS-KP, and showing significant inhibition in fish exposed to S(+)-KP. Several hypotheses could explain this discrepancy, such as post-transcriptional stabilization of mRNA, translational delay or inversion of response (Di Bello et al., 2007; Nahrgang et al., 2010). In particular, an *in vitro* study showed that maximal induction of EROD occurs after 6 h of exposure period to RS-KP, while an increase in exposure time to 12 h, did not produce EROD induction (Thibaut and Porte, 2008). As mentioned above, our study focused on a long-term exposure in order to mimic the environmental chronic exposure and thus, it is possible that extended exposure inhibited the EROD activity. However, we can also not exclude that the lower activities registered could be a consequence of a rapid metabolization of these drugs *via* other detoxifying pathways.

Another important Cyp enzyme involved in drug metabolism is Cyp3a and in fish, it can be induced by a variety of compounds. The *cyp3a* expression is thought to be mediated by the pregnane X receptor (PXR) in Atlantic salmon (Finn, 2007; Uno et al., 2012). PXR regulates expression of many CYP isoforms and is activated by several xenobiotics including pharmaceuticals, although the specific mechanism of interaction is not well understood (Popovic et al., 2015). Our results suggest that both the racemate and his enantiomer can induce *cyp3a* gene and its translation to protein in the liver, thus implicating the activation of PXR. PCA analysis showed strong relationship between *cyp3a* and MROD activity at the highest concentrations of RS-KP, while *cyp1a* expression was more related to S(+)-KP. Overall, biotransformation data in liver pointed to the conclusion that there was no evidence of any biotransformation enzymatic activities, even though induction at both transcriptional and translational level of CYPs occurred. The earlier response at transcriptional and translational level compared to catalytic activity could be explained by the timing of post-translational processes. Previous studies on biotransformation enzymes in organisms exposed to different chemicals has reported this type of discrepancies between transcriptional and catalytic levels (Regoli et al., 2005; Benedetti et al., 2007, 2009). Specifically, the response discrepancies between mRNA and functional products (enzymes and proteins) observed for the biotransformation and antioxidant responses may imply that under stressful conditions produced by NSAIDs, an induced mRNA transcription may not be necessarily needed, in order to maintain metabolic equilibrium at physiological levels. The argument for this is that mRNA, proteins and enzymatic activities could be target of transcriptional, post-transcriptional and/or post-translational toxicity that include, but not limited to reduced mRNA stability, slower protein synthesis, folding alterations, and cofactor depletion. Therefore, given that cells may initiate transcriptional processes towards adaptation to stressors, a reduction in functional response might limit cellular capacity to adapt to

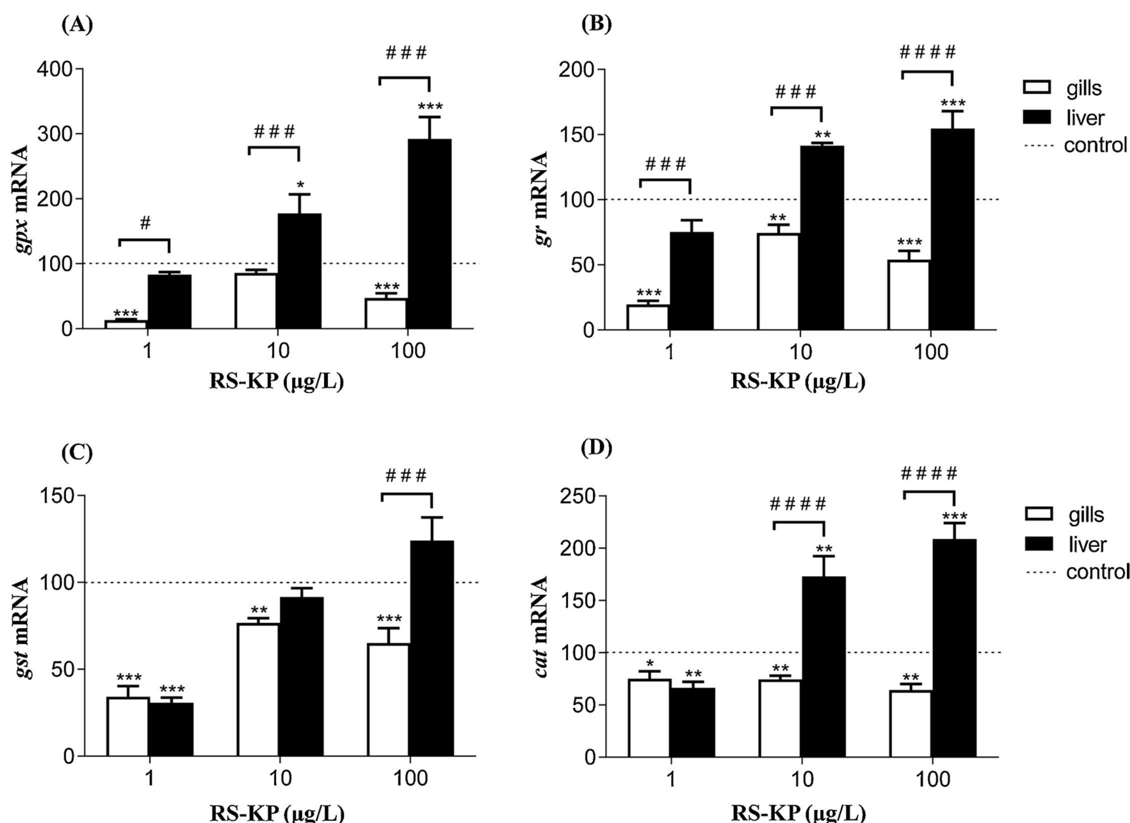

Fig. 4. Transcriptional changes of *gpx* (A), *gr* (B), *gst* (C) and *cat* (D) in gills and liver of juvenile of Atlantic salmon after 10 days of exposure to RS—KP. Data are reported as mean ± standard error of the mean (SEM) and presented as percent (%) of control (n = 6). Significant differences between control and exposure groups in each organ are reported as *$p \leq 0.05$; **$p \leq 0.01$ and ***$p \leq 0.001$ (One-way ANOVA followed by Dunnett's multiple comparison test) and between organs at the same concentration are marked with $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ and $^{\#\#\#\#}p \leq 0.0001$ (Two-way ANOVA followed by Sidak's multiple comparison test).

physiological changes resulting from exposure to NSAIDs, as has been previously demonstrated with estrogenic and anti-estrogenic responses (Celander, 2011; Arukwe et al., 2008) and sex-related differences in biotransformation responses (Larsen et al., 1992; Arukwe and Goksøyr, 1997). However, a clear interpretation necessitates the determination of expression at deepest level prior to defining a possible regulatory mechanism.

CYPs expression was also evaluated in the gills, as they represent the first physical barrier between the water compartment and fish. The gills are relatively sensitive to water quality variations (Poleksic and Mitrovic-Tutundzic, 1994; Fernandes and Mazon, 2003). Our data showed that the gills responded with consistent inhibition of *cyp1a* and *cyp3a* mRNA that parallel EROD and MROD activities. Additionally, PCA analysis confirmed similar effect from exposure to both drugs on gills, showing adjacent convex hulls located on the same side of the PCA diagram. Inhibitory responses measured in the gills was also observed in other environmental studies, where some categories of pharmaceuticals produced specific CYP inhibition in fish gills (Beijer et al., 2010). Different molecular and biochemical responses between tissues are due to their different metabolic capacity (Regoli et al., 2011), and in this case, it can also be hypothesized that a delayed response in the liver compared to the gills occurred.

4.2. Antioxidant responses

Previous studies in fish have reported increases in ROS production following exposure to pharmaceuticals, including NSAIDs (Li et al., 2010a, b; SanJuan-Reyes et al., 2013; Gonzalez-Rey and Bebianno, 2012). In particular, some classes of drugs can be metabolized by CYPs to active metabolites, that further induce associated-oxidative stress. Among antioxidants, Cat is a crucial enzyme which plays an important role by breaking down hydrogen peroxide (H_2O_2) and maintaining the cellular redox homeostasis. In our study, exposure to RS-KP and S(+)-KP induced a significant increase of liver *cat* mRNA. Other transcripts related to ROS detoxification (Gpx, Gr and Gst) were increased in the liver in response to high RS-KP and S(+)-KP exposures, whilst their transcripts were decreased in the gills. This is in accordance with previous reports Gonzalez-Rey and Bebianno, 2012), suggesting that exposure to RS-KP or S(+)-KP may affect the capacity to form reduced glutathione (GSH) in the gills. Furthermore, the different responses observed in the liver and gills enforce the idea that antioxidants may play diverse functions in different organs (Regoli et al., 2003). Indeed, in the literature, there are several reports showing how the antioxidant system can be modulated through either transient or permanent variation in different species, tissues and contaminant types (Regoli et al., 2003; Osman et al., 2007; Xiao et al., 2007; Binelli et al., 2009). The analysis of oxidative stress markers upon exposure to the racemate and its enantiomer, highlighted similar responses between the two molecules in both the liver and gills. However, the PCA analysis indicated that a more significant correlation exists between activation of GSH related oxidative markers and exposure to S(+)-KP in liver, while Cat is more related to RS-KP.

Additionally, a significant reduction of catalytic activities, compared to the control was observed in the gills after exposure to the highest concentration of RS—KP and S(+)-KP, in line with transcript expression levels. On the other hand, we observed activities similar to the control group in the liver. Interestingly, a clear transcriptional activation of the related antioxidant enzymes was also highlighted, suggesting that post-transcriptional mechanisms may represent an extra layer of regulation for the antioxidant defences (Regoli et al., 2003). A recent study that exposed adult zebrafish to RS—KP concentrations for 42 days, showed strong significant decreases of several hepatic antioxidant enzymes and

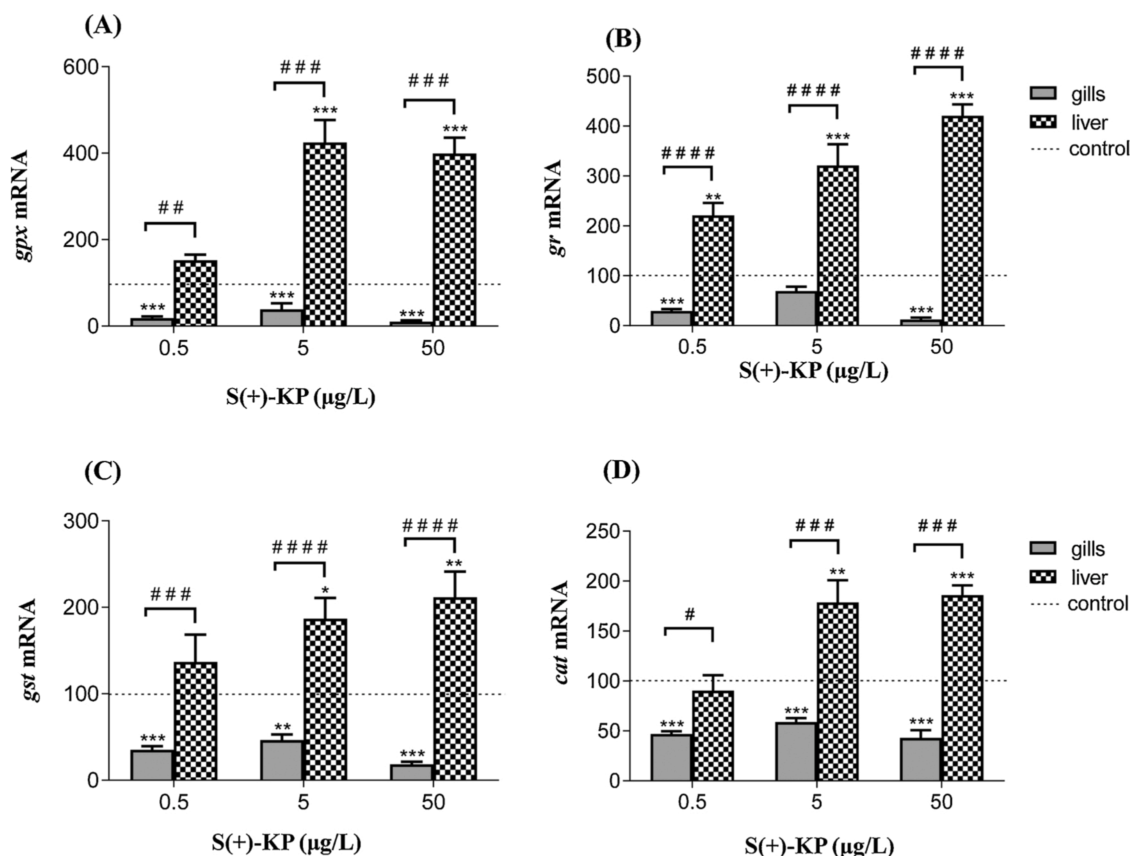

Fig. 5. Transcriptional changes of *gpx* (A), *gr* (B), *gst* (C) and *cat* (D) in gills and liver of juvenile of Atlantic salmon after 10 days of exposure to S(+)-KP. Data are reported as mean ± standard error of the mean (SEM) and presented as percent (%) of control (n = 6). Significant differences between control and exposure groups in each organ are reported as $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ (One-way ANOVA followed by Dunnett's multiple comparison test) and between organs at the same concentration are marked with $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$ and $^{\#\#\#\#}p \leq 0.0001$ (Two-way ANOVA followed by Sidak's multiple comparison test).

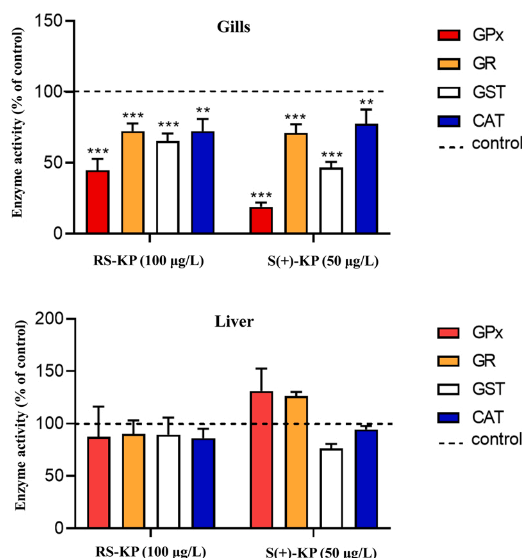

Fig. 6. Enzymatic activities of GPx, GR, GST and CAT in gills (A) and liver (B) of juvenile of Atlantic salmon after 10 days of exposure to highest concentration of RS—KP (100 μg/L) and S(+)-KP (50 μg/L). Data are reported as mean ± standard error of the mean (SEM) and presented as percent (%) of control (n = 6). Significant differences between control and exposure groups in each organ are reported as $^{*}p \leq 0.05$; $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ (One-way ANOVA followed by Dunnett's multiple comparison test).

associated histological damage (Rangasamy et al., 2018). In light of these and our findings, we may hypothesize that approximately 10 days of chronic exposure to NSAIDs is sufficient to severely impair antioxidant responses in the gills, as this organ represents the interface between contaminants and environment.

Subsequently, prolonged exposure (>10 days) to NSAIDs may have the potential to induce impairment and damage to other organs such as the liver. Indeed, further studies, aimed at investigating histological damage after chronic (>10 days) exposure to RS-KP and S(+)-KP are needed to confirm this hypothesis. Even though some differences were observed comparing the racemate to its enantiomer, showing pronounced effects when fish were exposed to S(+)-KP, our *in vivo* data did not highlight overall differential responses. This suggests that compound stereo-dependent toxicity may not be an issue in whole organisms with intact metabolic capacity. Nevertheless, more studies that include the R (-)-KP enantiomer may help in providing support for this assumption.

In conclusion, the present study has demonstrated the differences between RS-KP and S(+)-KP on toxicological responses related to biotransformation and antioxidant defence systems of fish. Interestingly, the exposure of fish to these NSAIDs produced significant, but differing effects on the liver and gills, and demonstrating a mode of action (MOA) that differentially targets toxicological and protective cellular responses at both transcriptional and functional product levels. Thus, our study has provided a solid overview and understanding on the molecular and cellular MOA for NSAIDs and possibly other pharmaceuticals that are ubiquitously distributed in environment.

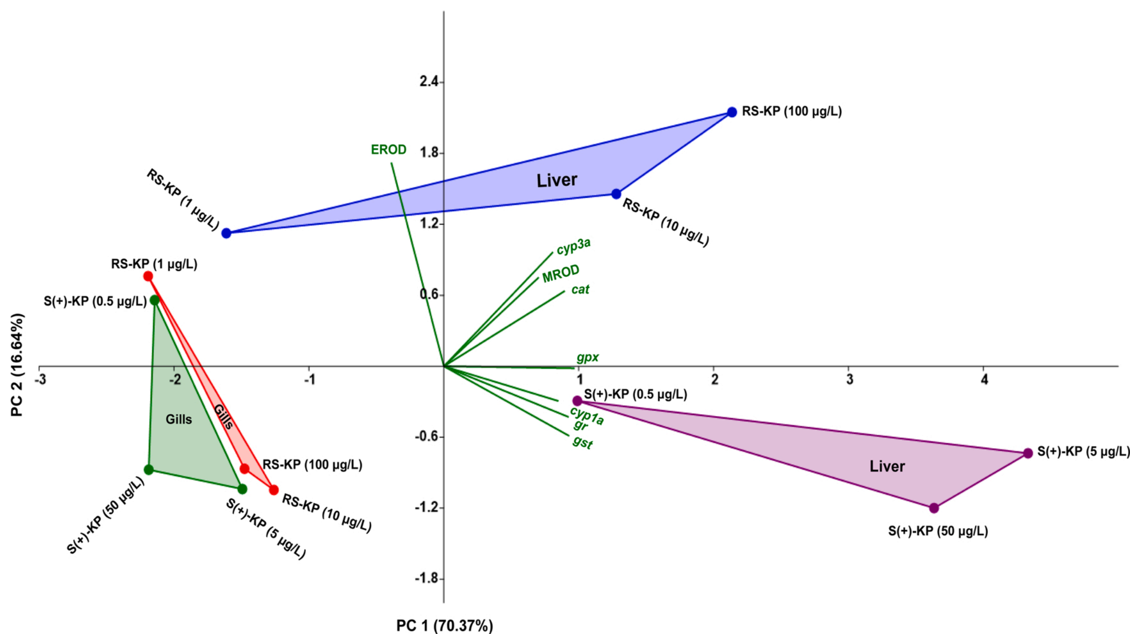

Fig. 7. Principal Component Analysis (PCA) diagram of measured biological markers in gills and liver of juvenile Atlantic salmon following exposure to RS-KP and S (+)-KP, two-dimensionally on x-y scatter plot, with combined principal component 1 and 2 (PC1 and PC2) accounted for 87 % of total variance. Coloured convex hulls defined each set of variance.

CRediT authorship contribution statement

Elvira Mennillo: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Carlo Pretti:** Writing - review & editing. **Francesca Cappelli:** Formal analysis, Writing - review & editing. **Giacomo Luci:** Formal analysis, Writing - review & editing. **Luigi Intorre:** Formal analysis, Writing - review & editing. **Valentina Meucci:** Formal analysis, Writing - review & editing. **Augustine Arukwe:** Conceptualization, Investigation, Supervision, Resources, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest associated with our study and paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105677.

References

Agranat, I., Caner, H., Caldwell, J., 2002. Putting chirality to work: the strategy of chiral switches. Nat. Rev. Drug Discov. 1, 753–768.
Andersson, T., Pärt, P., 1989. Benzo[a]pyrene metabolism in isolated perfused rainbow trout gills. Mar. Environ. Res. 28 (1-4), 3–7.
Anzenbacher, P., Anzenbacherová, E., 2001. Cytochromes P450 and metabolism of xenobiotics. Cell. Mol. Life Sci. 58, 737–747.
Arukwe, A., Goksøyr, A., 1997. Changes in three hepatic cytochrome P450 subfamilies during a reproductive cycle in turbot (*Scophthalmus maximus L.*). Endocrinology. 277 (4), 313–325.
Arukwe, A., Nordtug, D., Kortner, T.M., Mortensen, A.S., Brakstad, O.G., 2008. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (*Danio rerio*) exposed to water-soluble fraction of crude oil. Environ. Res. 107 (3), 362–370.
Asghar, A., Raman, A.A.A., Daud, W.M.A.W., 2015. Advanced oxidation processes for *in-situ* production of hydrogen peroxide/hydroxyl radical for textile wastewater treatment: a review. J. Clean. Prod. 87 (15), 826–838.
aus der Beek, T., Weber, F.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küst, A., 2016. Pharmaceuticals in the environment – global occurrences and perspectives. Environ. Toxicol. Chem. 35 (4), 823–835.
Barbanoj, M.J., Antonijoan, R.M., Gich, I., 2001. Clinical pharmacokinetics of dexketoprofen. Clin. Pharmacokinet. 40 (4), 245–262.
Beijer, K., Abrahamson, A., Brunström, B., Brandt, I., 2010. CYP1A inhibition in fish gill filaments: a novel assay applied on pharmaceuticals and other chemicals. Aquat. Toxicol. 96 (2), 145–150.
Benedetti, M., Martuccio, G., Fattorini, D., Canapa, A., Barucca, M., Nigro, M., Regoli, F., 2007. Oxidative and modulatory effects of trace metals on metabolism of polycyclic aromatic hydrocarbons in the Antarctic fish *Trematomus bernacchii*. Aquat. Toxicol. 85, 167–175.
Benedetti, M., Fattorini, D., Martuccio, G., Nigro, M., Regoli, F., 2009. Interactions between trace metals (Cu, Hg, Ni, Pb) and 2,3,7,8-tetrachlorodibenzo-p-dioxin in the antarctic fish *Trematomus bernacchii*: oxidative effects on biotransformation pathway. Environ. Toxicol. Chem. 28, 818–825.
Binelli, A., Parolini, M., Cogni, D., Pedriali, A., Provini, A., 2009. A multi-biomarker assessment of the impact of the antibacterial trimethoprim on the non-target organism Zebra mussel (*Dreissena polymorpha*). Comp. Biochem. Physiol. C. 150, 329–336.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. Anal. Biochem. 72, 248–254.
Burke, M.D., Mayer, R.T., 1974. Ethoxyresorufin: direct fluorometric assay of a microsomal O- dealkylation which is preferentially inducible by 3-methylcholanthrene. Drug Metab. Dispos. 2, 583–588.
Caballo, C., Sicilia, M.D., Rubio, S., 2015. Enantioselective analysis of nonsteroidal anti-inflammatory drugs in freshwater fish based on microextraction with a supramolecular liquid and chiral liquid chromatography-tandem mass spectrometry. Anal. Bioanal. Chem. 407, 4721–4731.
Celander, M.C., 2011. Cocktail effects on biomarker responses in fish. Aquat. Toxicol. 105 (3–4), 72–77.
Di Bello, D., Vaccaro, E., Longo, V., Regoli, F., Nigro, M., Benedetti, M., Gervasi, P.G., Pretti, C., 2007. Presence and inducibility by β-naphthoflavone of CYP1A1, CYP1B1 and phase II enzymes in Trematomus bernacchii, an Antarctic fish. Aquat. Toxicol. 84, 19–26.
Dorne, J.L.C.M., Skinner, L., Frampton, G.K., Spurgeon, D.J., Ragas, A.M.J., 2007. Human and environmental risk assessment of pharmaceuticals: differences, similarities, lessons from toxicology. Anal. Bioanal. Chem. 37, 1259–1268.
Fent, K., Weston, A.A., Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76, 122–159.
Fernandes, M.N., Mazon, A.F., 2003. Environmental pollution and Fish Gill morphology. In: Val, A.L., Kapoor, B.G. (Eds.), Fish Adaptation. Science Publishers, Enfield, pp. 203–231.
Finn, R.N., 2007. The physiology and toxicology of salmonid eggs and larvae in relation to water quality criteria. Aquat. Toxicol. 81, 337–354.
Gonzalez-Rey, M., Bebianno, M.J., 2012. Does non-steroidal anti-inflammatory (NSAID) ibuprofen induce antioxidant stress and endocrine disruption in mussel *Mytilus galloprovincialis*? Environ. Toxicol. Pharmacol. 33 (2), 361–371.
Gworek, B., Kijenska, M., Zaborowska, M., Wrzosek, J., Tokarz, L., Chmielewski, J., 2019. Pharmaceuticals in aquatic environment. Fate and behavior, ecotoxicology and risk assessment – a review. Acta Pol. Pharm. 76 (3), 397–407.

Herberer, T., 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131, 5–17.
Hong, J., Yang, C.S., 1997. Genetic polymorphism of cytochrome P450 as a biomarker of susceptibility to environmental toxicity. Environ. Health Perspect. 105 (4), 759–762.
Illés, E., Szabò, E., Takàcs, E., Wojnàrovits, L., Dombi, A., Gajda-Schrantz, K., 2014. Ketoprofen removal by O3 and O3\UV processes: kinetics, transformation products and ecotoxicity. Sci. Tot. Environ 472, 178–184.
IWW, 2014. Pharmaceuticals in the Environment: Occurrence, Effects, and Options for Action. Research Project Funded by German Federal Environment Agency (UBA) Within the Environmental Research Plan No. 3712 65 408.
Jones, O.A., Voulvoulis, N., Lester, J.N., 2001. Human Pharmaceuticals in the Aquatic Environment - a Review. Environ. Technol. 22 (12), 1383–1394.
Jönsson, H., Schiedek, D., Grøsvik, B.E., Goksøyr, A., 2006. Protein responses in blue mussels (*Mytilus edulis*) exposed to organic pollutants: a combined CYP-antibody/proteomic approach. Aquat. Toxicol. 78 (1), 49–56.
Kafaei, R., Papari, F., Seyedabadi, M., Sahebi, S., Tahmasebi, R., Ahmadi, M., Sorial, G. A., Asgari, G., Ramavandi, B., 2018. Occurrence, Distribution, and Potential Sources of Antibiotics Pollution in the Water-Sediment of the Northern Coastline of the Persian Gulf, Iran. Sci. Total Environ. 627, 703–712.
Kroon, F., Streten, C., Harries, S., 2017. A protocol for identifying suitable biomarkers to assess fish health: a systematic review. PLoS One 12 (4), e017476.
Kulcsár, A., Mátis, G., Petrilla, J., Neogrády, Zs., 2016. A bélnyálkahártya szerepe a xenobiotikumok biotranszformációjában, különös tekintettel a citokróm P450 enzimrendszerre. Irodalmi áttekintés. (the role of intestinal mucosa in the metabolism of xenobiotics with particular regard to cytochrome P450 enzyme system. Literature review). Magy. Allatorvosok Lapja 138, 243–250.
Kümmerer, K., 2013. Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks. Springer Science & Business Media, Berlin.
Lapworth, D.J., Baran, N., Stuart, M.E., Ward, R.S., 2012. Emerging organic contaminants in groundwater: a review of sources, fate and occurrence. Environ. Pollut. 163, 287–303.
Larsen, H.E., Celander, M., Goksøyr, A., 1992. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. Fish Physiol. Biochem. 10, 291–301.
Levine, S.L., Oris, J.T.C., 1999. YP1A expression in liver and gill of rainbow trout following waterborne exposure: implications for biomarker determination. Aquat. Toxicol. 46 (3–4), 279–287.
Li, Z.H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010a. Effects of exposure to sublethal propiconazole on the antioxidant defense system and Na+ K+-ATPase activity in brain of rainbow trout, Oncorhynchus mykiss. Aquat. Toxicol. 98, 297–303.
Li, Z.H., Zlabek, V., Grabic, R., Li, P., Machova, J., Velisek, J., Randak, T., 2010b. Effects of exposure to sublethal propiconazole on intestine-related biochemical responses in rainbow trout, Oncorhynchus mykiss. Chem. Biol. Interact. 185, 241–246.
Maharajan, K., Muthulakshmi, S., Nataraj, B., Ramesh, M., Kadirvelu, K., 2018. Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (*Danio rerio*): a multi biomarker study. Aquat. Toxicol. 196, 132–145.
Marsik, P., Rezek, J., Zidkova, M., Kramulova, B., Tauchen, J., Vanek, T., 2017. Non-steroidal anti-inflammatory drugs in the watercourses of Elbe basin in Czech Republic. Chemosphere. 171, 97–105.
Mennillo, E., Arukwe, A., Monni, G., Meucci, V., Intorre, L., Pretti, C., 2018a. Ecotoxicological properties of ketoprofen and the s(+)-enantiomer (dexketoprofen): bioassays in freshwater model species and biomarkers in fish plhc-1 cell-line. Environ. Toxicol. Chem. 37 (1), 201–212.
Mennillo, E., Krøkje, Å., Pretti, C., Meucci, V., Arukwe, A., 2018b. Biotransformation and oxidative stress responses in rat hepatic cell-line (H4IIE) exposed to racemic ketoprofen (RS-KP) and its enantiomer, dexketoprofen (S (+)-KP). Environ. Toxicol. Pharmacol. 59, 199–207.
Mezzelani, M., Gorbi, S., Da Ros, Z., Fattorini, D., d'Errico, G., Milan, M., Bargelloni, L., Regoli, F., 2016. Ecotoxicological potential of non-steroidal anti-inflammatory drugs (NSAIDs) in marine organisms: bioavailability, biomarkers and natural occurrence in Myti*lus galloprovincialis*. Mar. Environ. Res. 121, 31–39.
Murray, K.E., Thomas, S.M., Bodour, A.A., 2010. Prioritizing research for trace pollutants and emerging contaminants in the freshwater environment. Environ. Pollut. 158, 3462–3471.
Nahrgang, J., Camus, L., Gonzalez, P., Jönsson, M., Christiansen, J.S., Hop, H., 2010. Biomarker responses in polar cod (*Boreogadus saida*) exposed to dietary crude oil. Aquat. Toxicol. 96, 77–83.
Nikolaou, A., Meric, S., Fatta, D., 2007. Occurrence patterns of pharmaceuticals in water and wastewater environments. Anal. Bioanal. Chem. 387 (4), 1225–1234.
Nunes, B., Antunes, S.C., Gomes, R., Campos, J.C., Braga, M.R., Ramos, A.S., Correia, A. T.A., 2015. cute Effects of Tetracycline Exposure in the Freshwater Fish *Gambusia holbrooki*: Antioxidant Effects, Neurotoxicity and Histological Alterations. Arch. Environ. Con. Tox. 68, 371–381.
Oris, J.T., Roberts, A.P., 2009. Statistical analysis of cytochrome P4501A biomarker measurements in fish. Environ. Toxicol. Chem. 26 (8), 1742–1750.
Osburn, W.O., Kensler, T.W., 2008. Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. Mutat. Res. 659 (1-2), 31–39.
Osman, A.M., van den Heuvel, H., van Noort, P.C.M., 2007. Differential responses of biomarkers in tissues of a freshwater mussel, *Dreissena polymorpha*, to the exposure of sediment extracts with different levels of contamination. J. Appl. Toxicol. 27, 51–59.
Pesonen, M., Andersson, T., 1987. Subcellular localization and properties of cytochrome P-450 and UDP glucuronosyltransferase in the rainbow trout kidney. Biochem. Pharmacol. 36, 823–829.
Poleksic, V., Mitrovic-Tutundzic, V., 1994. Fish gills as a monitor of sublethal and chronic effects of pollution. In: MÜLLER, R., LLOYD, R. (Eds.), Sublethal and Chronic Effects of Pollutants on Freshwater Fish. Cambridge, pp. 339–352.
Popovic, N.T., Klobucar, R.S., Strunjak-Perovic, I., Jadan, M., Barisic, J., Cos-Rakovac, R., 2015. Piscine cytochromes P450 (CYP) and their response to antimicrobial drugs. Aquac. Res. 46 (2), 257–271.
Rangasamy, B., Hemalatha, D., Shobana, C., Nataraj, B., Ramesh, M., 2018. Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to anti-inflammatory drug ketoprofen. Chemosphere 213, 423–433.
Regoli, F., Winston, G.W., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I., et al., 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*. Environ. Toxicol. Chem. 22, 2120–2129.
Regoli, F., Nigro, M., Benedetti, M., Gorbi, S., Pretti, C., Gervasi, P.G., Fattorini, D., 2005. Interactions between metabolism of trace metals and xenobiotic agonists of the aryl hydrocarbon receptor in the Antarctic fish Trematomus bernacchii: Environmental Perspectives. Environ. Toxicol. Chem. 24, 1475–1482.
Regoli, F., Giuliani, M.E., Benedetti, M., Arukwe, A., 2011. Molecular and biochemical biomarkers in environmental monitoring: a comparison of biotransformation and antioxidant defense systems in multiple tissues. Aquat. Toxicol. 105S, 56–66.
SanJuan-Reyes, N., Gómez-Oliván, L.M., Galar-Martínez, M., VieyraReyes, P., García-Medina, S., Islas-Flores, H., Neri-Cruz, N., 2013. Effluent from an NSAID-manufacturing plant in Mexico induces oxidative stress on *Cyprinus carpio*. Water Air Soil Pollut. 224, 1689.
Spongberg, A.L., Witter, J.D., Acuña, J., Vargas, J., Murillo, M., Umaña, G., Gómez, E., Perez, G., 2011. Reconnaissance of selected PPCP compounds in Costa Rican surface waters. Water Res. 45, 6709–6717.
Thibaut, R., Porte, C., 2008. Effects of fibrates, anti-inflammatory drugs and antidepressants in the fish hepatoma cell line PLHC-1: cytotoxicity and interactions with cytochrome P450 1A. Toxicol. In. Vitro. 22, 1128–1135.
Trudeau, V.L., Metcalfe, C.D., Mimeault, C., Moon, T.W., 2005. Chapter 17 Pharmaceuticals in the environment: Drugged fish? Biochem. Mol. Biol. Fish. 6, 475–493.
Uno, T., Ishizuka, M., Itakura, T., 2012. Cytochrome P450 (CYP) in fish. Environ. Toxicol. Pharm. 34 (1), 1–13.
Weigel, S., Berger, U., Jensen, E., Kallenborn, P., Thoresen, H., Hühnerfuss, H., 2004. Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites. Chemosphere. 56, 583–592.
Williams, R.T., 2005. Human Pharmaceuticals: Assessing the Impacts on Aquatic Ecosystems. The Society of Environmental Toxicology and Chemistry, FL, USA.
Xiao, Q., Zhang, S., Guo, H., Su, F., Xu, Y., 2007. Nonylphenol causes decrease in antioxidant enzyme activities, increase in O2 - content, and alterations in ultrastructures of FG cells, a Flounder (*Paralichthys olivaceus*) gill cell line. Toxicol. Mech. Met. 17, 127–134.
Yin, L., Wang, B., Yuan, H., Deng, S., Huang, J., Wang, Y., et al., 2017. Pay special attention of transformation products of PPCPs in environment. Emerg. Contam. 3, 69–75.