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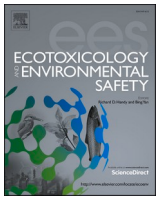

Bioaccumulation and biochemical responses in the peppery furrow shell *Scrobicularia plana* exposed to a pharmaceutical cocktail at sub-lethal concentrations

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ABSTRACT

Pharmaceutical drugs in the aquatic medium may pose significant risk to non-target organisms. In this study, the potential toxicity of a mixture of three compounds commonly detected in marine waters (ibuprofen, ciprofloxacin and flumequine) was assessed, by studying bioaccumulation, oxidative stress and neurotoxicity parameters (catalase CAT, superoxide dismutase SOD, glutathione reductase GR, glutathione S-transferase GST, lipid peroxidation LPO, glutathione peroxidase GPX, metallothionein MT and acetylcholinesterase AChE) in the clam *Scrobicularia plana*. Temporal evolution of selected endpoints was evaluated throughout an exposure period (1, 7 and 21 days) followed by a depuration phase. The accumulation of all drugs was fast, however clams showed the ability to control the internal content of drugs, keeping their concentration constant throughout the exposure and reducing their content after 7 days of depuration. The induction of biochemical alterations (SOD, CAT, LPO, MT, AChE) was observed in gills and digestive gland probably related to an imbalance in the redox state of clams as a consequence of the exposure to the drug mixture. These alterations were also maintained at the end of the depuration week when the high levels of SOD, CAT, GST and LPO indicated the persistence of oxidative stress and damage to lipids despite the fact that clams were no longer exposed to the mixture.

1. Introduction

In the last decades a wide range of pharmaceuticals have been detected in aquatic matrices around the world. Urban domestic effluents, hospital effluents and animal husbandry are considered the main emission sources; the continuous release from point and non-point sources of a wide range of compounds produces in the aquatic environment a "complex pharmaceutical pool" (approximately 20,000 prescription drugs and 1600 veterinary products are approved by the US Food and Drug Administration and almost 3000 biologically active compounds are identified in the environment) (Reyes et al., 2021). The pharmaceutical concentrations in surface waters (both fresh and marine waters) generally range from a few pg L^{-1} to several μg L^{-1} (Mezzelani et al., 2018). These compounds are biologically active and cause specific effects even at low doses. High bioactivity and pseudo-persistence in the aquatic medium, due to specific physico-chemical properties as well as the continuous discharge, can lead to a chronic exposure of aquatic organisms with consequent bioaccumulation and induction of sublethal effects whose knowledge, despite the growing interest of the scientific community, remains one of the biggest gaps in pharmaceutical research.

Laboratory studies carried out with individual compounds at environmentally realistic concentrations recognize the ability of pharmaceuticals to induce a wide range of toxic effects in exposed organisms; molecular, biochemical and cellular alterations (gene expression modulation, genotoxic damage, immunological alterations, modulation of detoxification pathways, alteration of oxidative status, among others), morphological and tissue changes, impairment of biological processes (reproduction, growth, metabolic processes, feeding, locomotion) have been documented in mollusks, crustaceans, fish, polychaete and phytoplankton (Antunes et al., 2013; Correia et al., 2016; Chia et al.,

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2021; Curpan et al., 2022; Matozzo et al., 2012a; Mezzelani et al., 2018; Swiacka et al., 2020). The effects produced by drug mixtures have also been studied showing the occurrence of interactions between the chemicals present in the mixture and consequently different effects than those expected (Beghin et al., 2021; Godoy and Kummrow, 2017). However, the studies focusing on mixtures are much scarcer than studies on the toxicity of individual compounds, despite the fact that the former better reflect what happens in the real aquatic environment.

Antibiotics and anti-inflammatory/analgesic drugs are currently at the leading position among the environmental pollutants. Ciprofloxacin (CIP) and flumequine (FMQ) are synthetic broad-spectrum fluoroquinolone antibiotics increasingly used in human and veterinary applications respectively and detected in the aquatic environment in a wide range of concentrations (CIP: 0.0015–6500 μg L^{-1} in surface waters including fresh and seawater systems, 8–31 mg L^{-1} in effluents from hospitals and wastewater treatment plants (WWTP); FMQ: 3–192 ng L^{-1} in rivers, 2.44–41 ng L^{-1} in WWTP effluents, Danner et al., 2019; Kovalakova et al., 2020; Liu et al., 2018). Fluoroquinolones have been generally found to be capable of causing toxic effects (oxidative stress, genotoxicity, neurotoxicity) in algae, bacteria, plants, crustaceans and fishes (Sodhi and Singh, 2021; Yang et al., 2020). On the other hand, ibuprofen (IBU) is one of the main non-steroidal anti-inflammatory drugs used worldwide and, consequently, one of the most detected pharmaceuticals in aquatic ecosystems; IBU concentrations vary from few ng L^{-1} (0.02–10053 in surface waters) to μg L^{-1} (35–151 in hospital and WWTP effluents) and its chronic toxicity is well documented in a variety of aquatic organisms (Almeida et al., 2020; Majumder et al., 2019; Parolini, 2020; Wang et al., 2021 among others).

In this study, the potential impact of the mixture of CIP, FMQ and IBU was analyzed in the peppery furrow shell clam *Scrobicularia plana* (da Costa, 1778), starting from the hypothesis that, as filter feeding organism, it can uptake pharmaceuticals from the water medium, accumulate them in its tissues and consequently activate mechanisms to counteract the possible adverse effects induced by these drugs. Two sublethal concentrations, chosen within the environmental range for the compounds, were tested, the first one (10 μg L^{-1}) close to environmentally relevant concentrations and the other tenfold higher (100 μg L^{-1}), representative of highest contamination levels and supposedly capable of inducing distinguishable responses in the organisms. Molecular and biochemical responses are sensitive and tend to occur in early stages of exposure; thus, by monitoring their changes we can detect the potential harm of chemical stressors. Multiple toxicological endpoints were selected in this study to assess the consequences of pharmaceutical mixture's exposure in clams: activity/level of enzymes and proteins involved in the antioxidant and detoxification systems (catalase CAT, superoxide dismutase SOD, glutathione reductase GR, total and Se-dependent glutathione peroxidase T- and Se-GPx, glutathione S-transferase GST, metallothioneins MTs), activity of acetylcholinesterase (AChE) an enzyme commonly used as biomarker of neurotoxicity and that could be involved in defense mechanisms against xenobiotics in invertebrates (Kim and Lee, 2018), and levels of lipid peroxidation (LPO) as indicator of oxidative damage in clams. The work focused on the temporal evolution of the selected sublethal responses over 21 days of exposure to the drug mixture to assess the health status progression of clams. Moreover, we also checked the responses (both biomarkers and drugs accumulation) of clams after a depuration phase of 7 days in a clean aquatic medium following exposure, in order to evaluate their ability to overcome possible biological alterations and to eliminate the accumulated drugs.

2. Materials and methods

2.1. Laboratory exposure assay

Specimens of *S. plana* (2.6 ± 0.2 cm shell length) were collected in autumn (end of October) from the Rio San Pedro intertidal mudflat (36°32′00.1′'N, 6°12′51.9′'W, Cádiz, SW Iberian Peninsula) considered as a low contaminated area (Biel-Maeso et al., 2018). They were transferred to the laboratory and acclimated for 2 weeks in a flow-through seawater system (filtered to 0.45 μm) with constant aeration and natural photoperiod.

Exposure was carried out in tanks filled with filtered seawater (0.45 μm) and constant aeration, under natural photoperiod and semi-static conditions. The ratio seawater volume/number of clams in each tank was maintained constant 0.4 L/organism and remained constant throughout the experiment. Pharmaceuticals (CAS 85721-33-1, 42835-25-6 and 15687-27-1 respectively for CIP, FMQ and IBU, all purchased from Sigma Aldrich, Germany, purity ≥98 %, main characteristics shown in Supplementary Material S1) were added to the exposure tanks as a stock solution prepared in DMSO (final DMSO concentration in the tanks: 0.002 % v/v). Three experimental conditions were tested simultaneously, each one in triplicates (three tanks for each experimental condition): DMSO control, low and high mixture concentration (respectively 10 and 100 μg L^{-1} for each compound present in the mixture).

Clams were randomly added to each tank (145 organisms/tank) and exposure run for 21 days, followed by 7 days of depuration. Dissolved oxygen, temperature, pH and salinity were monitored daily (8.8 ± 0.6 mg L^{-1}, 18.0 ± 1.5 °C, 7.9 ± 0.4 and 31.0 ± 0.3 respectively) and water was changed every 48 h and spiked according to the nominal concentrations. During both acclimation and exposure, clams were fed with commercial liofilized food (TropicMarin Pro-coral PHYTON) every 48 h (2 measuring spoons of food in each tank), 2 h before water renovation.

Individuals were randomly collected after 1, 7, 21 and 28 days (14 clams from each tank at each sampling time). After collection, the 14 clams were distributed at random into two groups according to the following: 10 organisms (whole body) were individually used for pharmaceutical quantification and 4 clams were dissected (digestive gland and gills) and tissues pooled for biomarker analysis. The whole bodies and the pools were immediately frozen in liquid nitrogen and stored at −80 °C for later analysis.

Water sampling was performed to check pharmaceutical concentrations in the tanks. During the exposure phase, water samples (10 mL) from each tank were collected after stock solution addition and 48 h later (before the next exposure medium renovation and before adding the food to the tanks); water samples were taken on days 0, 2, 6, 8, 14, 16. During the depuration phase samples (10 mL from each tank) were taken on the day 23.

Clam mortality was daily checked.

2.2. Pharmaceutical quantification in clams and exposure medium

CIP, FMQ and IBU were quantified by ULPC QTOF mass spectrometry in the soft tissues (whole body) of clams (n = 10 individual organisms analysed per tank and per time which means n = 30 organisms per treatment and time) and in water samples. Biological samples were lyophilized (LabconcoFreezone 2.3, MA, USA) and then extracted by applying microwave energy during 5 min and 50 W, (Ethos 900, Milestone). Lyophilized samples (0.1 g) were treated with 2 mL of a mixture acetonitrile: water 50:50 (v/v) spiked with 10 μL proteinase-K PCR recombinant (Roche Diagnosis, Barcelona, Spain) and 1 μL of formic acid. The obtained extracts were centrifuged at 9000 rpm for 10 min (Hettich Universal 320), followed by evaporation under N_2 stream until nearly dryness. Finally, the extract was reconstituted with 100 μL of 0.1 % formic acid aqueous solution and microfiltrated through 0.2 μm before LC-ESI-QTOF-MS injection.

Water samples were microfiltered (0.2 μm) and a 10 μL aliquot was directly injected into the chromatographic system. Samples from each tank were analyzed in duplicates.

Pharmaceutical detection was carried out by LC-ESI-QTOF-MS (Xevo G2-S Waters Corporation, Mildford, MA, USA) in full scan resolution mode and ESI positive/negative mode was applied for ionization of the

analytes using an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters Corporation, Mildford, MA, USA). The mobile phase was water (A) and acetonitrile (0.1 % formic acid) (B). A gradient elution was applied for ten minutes consisting in 90 % A initially to 45 % A in 4.5 min, a subsequent isocratic step for 3.5 min returning to initial conditions at t = 8.0 min. Two minutes were waited for reequilibration before the following injection. The flow rate was set at 0.3 mL min^{-1}. CIP, FMQ and IBU analytical standards (from Sigma-Aldrich, Merck Life Science S.L.U., Spain) were used for standard curves. Limits of quantification (LOQs) for the method were in the range of 2.81–3.61 ng g^{-1} for biological samples (CIP: 2.81, FMQ: 3.61, IBU: 2.86) and 2.01–2.81 ng L^{-1} for water samples (CIP: 2.01, FMQ: 2.81, IBU: 2.21). Instrumental limits of detection (LODs) were in the range of 0.73–1.39 ng g^{-1} (CIP: 0.73, FMQ: 1.39, IBU: 1.16) and 0.19–1.04 ng L^{-1} (CIP: 0.19, FMQ: 0.93, IBU: 1.04) for biological and water samples respectively.

2.3. Biochemical analysis

Gills and digestive gland tissues (pools constituted by tissues of 4 individuals, 3 pools per treatment) were homogenized on ice in 50 mM Tris buffer (150 mM NaCl, 1 mM DTT, 0.1 % antiproteolitic cocktail at pH 7.4) using an Ultra-turrax homogenizer (ULTRA-TURRAX T25, Janke&Kunkel, IKA®Labortechnich). The homogenate was then centrifuged at 12,000 x *g* at 4 °C during 30 min and the supernatant collected and divided in aliquots for the determination of SOD, CAT, T- and Se-GPx, GST, GR, LPO, MT and AChE as well as total protein concentrations.

Biochemical determinations were performed by spectrophotometry (microplate reader Tecane Infinite 200, Tecane Group Ltd, Austria).

Total protein content was determined according to Bradford's method (Bradford, 1976) using BSA as standard to normalize enzyme activities, LPO and MT levels.

SOD activity was assessed using a SOD Assay Kit-WST (Dojindo Laboratories, Japan) according to the method of Ukeda et al. (1999). SOD from bovine erythrocytes was used as standard. Activity was expressed as U SOD mg-1 protein where 1 U corresponds to the amount of the enzyme in the sample volume that causes 50 % inhibition activity of SOD).

CAT activity was evaluated by measuring the consumption of hydrogen peroxide (H_2O_2) at 230 nm according to the method described by Li and Schellhorn (2007). CAT activity was expressed as U CAT mg^{-1} protein where 1 U is defined as the amount of enzyme that causes the loss of 1.0 μmol of H_2O_2 per min, under the assay conditions.

T- and Se-GPx activities were measured at 340 nm following the loss of NADPH in presence of excess glutathione peroxidase, reduced glutathione and the corresponding peroxide (McFarland, 1999). Cumene peroxide and H_2O_2 were used as substrates for the determination of T- and Se-GPx activity, respectively. Activity was expressed as nmol NADPH min^{-1} mg^{-1} protein.

GST activity was determined following the method described by McFarland et al. (1999) using 1-chloro 2,4 dinitrobenzene (CDNB) as substrate. The increase in absorbance was recorded at 340 nm and results were reported as nmol CDNB min^{-1} mg^{-1} protein.

GR determination was conducted following the method of McFarland et al. (1999). The loss of NADPH present in the reaction mixture was recorded at 340 nm and activity was reported as nmol NADPH min^{-1} mg^{-1} protein.

LPO levels were quantified following the thiobarbituric acid reactive substances method (Hannam et al., 2010). Malondialdehyde (MDA) concentration, as final product of oxidative lipid degradation, was used to determine (absorbance measured at 535 nm) LPO levels and results were expressed as nmol MDA mg^{-1} protein.

AChE activity was determined using the method proposed by Ellman et al. (1961) and adapted to the microplate reader by Bocquené and Galgani (1998) in which reaction progress between acethylthiocholine (ACTC) and 5,5′-dithio-bis (2-nitrobenzoic acid) is followed by recording absorbance (405 nm) changes. Activity was expressed as nmol ACTC min^{-1} mg^{-1} protein.

Total MT content was determined in the supernatant by RP-HPLC coupled to fluorescence detection (W2695 Separation Module, 2475 Multi λ fluorescence Detector, WATERS Corporation, Milford, MA, USA) using rabbit liver MT-I as a reference for standard curve, according to the protocol developed by Alhama et al. (2006). Supernatant was derivatized with the fluorogenic reagent mBBr after treatment at 70 °C with EDTA, SDS and DTT. Final concentrations for DTT, EDTA, SDS and mBBr in a total volume of 100 μL were 10 mM, 2 mM, 3 % and 12 mM, respectively. Derivatized proteins were separated in a C18 X Bridge column (6 ×150 mm, 5 μm particle, WATERS Corporation, Milford, MA, USA) and fluorescence was monitored with excitation at 382 nm and emission at 470 nm (gain=10). MT content was expressed as μg MT mg^{-1} protein.

2.4. Data analysis

Statistical analyses were conducted using the statistical package R (R Core Team, 2017, https://www.R-project.org/). Statistical significance was established at $p < 0.05$.

Bioaccumulation and biochemical data were checked for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Leven's test).

In the case of bioaccumulation, significance ($p < 0.05$) of drug concentrations between days within each treatment (control, CIP 10 μg L^{-1}, CIP 100 μg L^{-1}, FMQ 10 μg L^{-1}, FMQ 100 μg L^{-1}, IBU 10 μg L^{-1} and IBU 100 μg L^{-1}), was tested using one-way analysis of variance (ANOVA) followed by Tukey test (when normality and homoscedasticity of data were verified) or the non-parametric Kruskall-Wallis followed by Dunn's post hoc test (when assumptions were not verified).

In the case of biomarker, we separately evaluated data for exposure and depuration phases. Significant differences ($p < 0.05$) between control and treatment groups (mixture at 10 and 100 μg L^{-1}) throughout the exposure time were evaluated using two-way ANOVA followed by Tukey post hoc test. Lastly, one-way ANOVA with a Tukey's test was performed on data for depuration phase to perform multiples ($p < 0.05$) comparisons between treatments.

The significance values for drug quantification in clams and biomarkers are presented in Tables S3–S5 (Supplementary Material). Bioconcentration factor (BCF) for each pharmaceutical compound was determined according to the following equation (Arnot and Gobas, 2006):

$$\frac{concentration\ in\ the\ clam\ whole\ body}{concentration\ in\ seawater}$$

For each tank, the numerator is the concentration of pharmaceutical compound in the clam whole body at the end of exposure (day 21) whereas the denominator is the average value of the concentrations recorded on the days of the stock solution in tanks (days 0, 6 and 14).

3. Results

The average mortality recorded throughout the experiment was 17.7 %. A higher (but no statistically significant) mortality percentage was observed at the highest mixture concentration (21.7 ± 4.6 %) compared to the control and the lowest mixture dose (15.2 ± 3.6 and 16.1 ± 3.6 % respectively).

3.1. Pharmaceuticals in exposure water and clams

The concentrations of FMQ, CIP and IBU measured during the exposure phase after addition of stock solution were slightly lower than expected (5–9 % lower in the case of the nominal concentration of 10 μg L^{-1} and 9 % in the case of 100 μg L^{-1}, Supplementary Material S2). Measured concentrations of pharmaceuticals decreased between 31

% and 45 % after 48 h of renewal of test solutions. All pharmaceuticals were detected in the exposure medium also during the depuration phase (water samples collected 48 h after starting depuration, Supplementary Material S2). Pharmaceuticals were not detected in control water samples.

Results of bioaccumulation analysis in the whole body of clams are shown in Table 1 and results of statistical analysis were presented in the Table S3 (Supplementary Material). The BCF values were reported for each compound and concentration at the end of the exposure phase (day 21). No pharmaceuticals were detected both in clams from acclimatization tanks and control organisms. A fast accumulation (1 day, Table 1) of all pharmaceuticals was observed at both exposure concentrations. FMQ levels in clams remained constant throughout the exposure phase (no significant differences between the days 1, 7 and 21 at both exposure doses, Table 1). Small but significant differences were observed for CIP between the exposure days (Table 1): CIP level for day 21 was significant different ($p < 0.05$) from days 1 and 7 in clams exposed to the mixture at low concentration whereas in the case of exposure to the pharmaceuticals at 100 μg L^{-1} significant difference ($p < 005$) was detected only between days 1 and 7. IBU concentrations in clams exposed to low drug dose did not present significant differences between the first and the last day of exposure, however a significant increase was recorded at the day 7 ($p < 0.05$, Table 1). In the case of exposure to the mixture at 100 μg L^{-1}, the IBU concentration for the day 21 was significantly lower compared to days 1 and 7 ($p < 0.05$, Table 1). The BCFs calculated at the end of the exposure (day 21) were similar for both exposure conditions (Table 1) varying between 0.82 and 0.98 at the low exposure dose and between 0.76 and 0.91 at the high dose. For organisms exposed to 10 μg drugs L^{-1}, the BCF values (although differences between them are low) indicate the following order of drug accumulation in the clam whole body: FMQ>CIP>IBU. For exposure to the highest mixture concentration BCF values for FMQ and CIP were very similar and higher than the value obtained for IBU. After one week of depuration the levels of pharmaceuticals accumulated in the whole body decreased significantly ($p < 0.05$) compared to the end of exposure phase (Table 1). The percentage of reduction was similar for all compounds and at both exposure concentrations (42 %, 44 % and 45 % for FMQ, CIP and IBU at 10 μg L^{-1} respectively, 44 % and 41 % for CIP and IBU respectively at 100 μg L^{-1}) with exception of FMQ which showed a reduction of 27 % compared to day 21.

Table 1
Flumequine (FMQ), ciprofloxacin (CIP) and ibuprofen (IBU) concentrations (ng g^{-1} wet weight, mean ± standard deviation, n = 30) in *Scrobicularia plana* (whole body) throughout the exposure assay at both experimental conditions (10 and 100 μg L^{-1}). For each compound statistically significant differences (Tukey, $p < 0.05$) between exposure days are indicated by letters. BCF values (L Kg^{-1}) corresponding to each compound at the end of exposure phase (day 21), were reported (mean ± standard deviation, n = 30). LOD: Limit of detection (FMQ: 1.39, CIP: 0.73, IBU: 1.16 ng g^{-1} wet weight).

Nominal concentration (μg L^{-1})	Exposure time (day)	Pharmaceutical concentration (ng g^{-1} wet weight)		
		FMQ	CIP	IBU
10	0	<LOD	<LOD	<LOD
	1	8.3 ± 0.1^{b}	8.2 ± 0.1^{c}	7.1 ± 0.4^{a}
	7	8.1 ± 0.9^{b}	7.9 ± 0.4^{c}	7.6 ± 0.2^{c}
	21	8.9 ± 1.0^{b}	8.2 ± 0.2^{b}	7.5 ± 0.2^{a}
	28	5.2 ± 0.2^{a}	4.6 ± 0.1^{a}	4.1 ± 0.2^{b}
	BCF_{21}	0.94 ± 0.10	0.84 ± 0.04	0.81 ± 0.02
100	0	<LOD	<LOD	<LOD
	1	76.4 ± 7.0^{a}	73.6 ± 5.9^{a}	68.8 ± 12.6^{a}
	7	81.6 ± 1.4^{a}	79.6 ± 2.7^{b}	75.6 ± 0.6^{a}
	21	83.4 ± 3.8^{a}	84.1 ± 3.0^{ab}	68.2 ± 2.9^{b}
	28	60.6 ± 6.4^{b}	46.9 ± 0.4^{c}	40.6 ± 2.2^{c}
	BCF_{21}	0.92 ± 0.04	0.93 ± 0.02	0.75 ± 0.03

3.2. Biochemical responses

The temporal evolution of biochemical biomarkers in digestive gland and gills is shown in the Figs. 1–3. Results of two-way ANOVA for biomarkers (significance of parameters and post-hoc comparisons) were presented in the Supplementary Material S4 and S5.

In digestive gland (Figs. 1 and 3A) the levels of SOD and AChE significantly increased ($p < 0.05$) during the first week of exposure to the mixture at 100 μg L^{-1}. At the end of the exposure phase neither of the two enzymes showed significant differences with respect to the control. Significant increase was also recorded for GST activity (100 μg L^{-1}, day 21, $p < 0.05$), LPO (100 μg L^{-1}, day 1, $p < 0.05$) and MT (10 and 100 μg L^{-1}, day 21, $p < 0.05$) levels (however, the two-way ANOVA does not relate these alterations to the exposure to the mixture, Supplementary Material S4). No significant alterations with respect to the control were observed in CAT, T-GPx, Se-GPx and GR. After the depuration phase a significant rise (with respect to the control) in the activity of GST (100 μg L^{-1}, $p < 0.05$) and SOD (10 μg L^{-1}, $p < 0.05$), and in LPO levels (10 μg L^{-1}, $p < 0.05$) was recorded in this tissue in organisms pre-exposed to the drug mixtures.

In gills, SOD activity significantly increased ($p < 0.05$) during the first week of exposure to the mixture at 10 μg L^{-1} (Fig. 2). Pairwise comparisons also indicated significant increase in AChE (day 7 and 21 in organisms exposed to 10 μg L^{-1} and only at day 21 in clams exposed to 100 μg L^{-1}, $p < 0.05$, Fig. 3B) activity. Additionally, changes in T-GPx (day 1, $p < 0.05$), Se-GPx (day 1, $p < 0.05$) and CAT (day 21, 100 μg L^{-1}, $p < 0.05$, Fig. 2) activities and MT (day 7, 100 μg L^{-1}, $p < 0.05$, Fig. 2) level were observed (however, these alterations are not related to the exposure mixture by two-way ANOVA, Supplementary Material S4). After a seven-day depuration phase we recorded a significant increase ($p < 0.05$) of CAT activity and LPO levels in organisms pre-exposed to both doses of pharmaceutical mixture.

4. Discussion

Despite the growing interest of the scientific community in the last decade for the impact of pharmaceuticals on aquatic organisms, knowledge on the effects of complex mixtures is still scarce. In this study, the clam *S. plana* was exposed to a mixture of FMQ, CIP and IBU at two different concentrations to evaluate the potential sublethal effects of the mixture. We observed accumulation of all compounds at both exposure doses. Accumulation was fast, reaching a maximum level within 24 h, and showed little (but in some cases significant) changes throughout the entire exposure phase. Similar results were observed in the same organism exposed to carbamazepine only (Almeida et al., 2017). A quick accumulation can be related to a high daily filtration rate (both by feeding through siphons and respiration by gills) and this is also reflected in the data from the drug quantification in the exposure medium; we observed a considerable decrease in FMQ, CIP and IBU concentrations 48 h after adding the stock solution (43 %, 45 % and 43 % respectively at the exposure dose of 10 μg L^{-1}, and 38 %, 34 % and 31 % at 100 μg L^{-1}). The observed constant level of drugs in clams during the exposure phase may indicate that organisms activate regulatory mechanisms that control the pharmaceutical accumulation among them an increased metabolization and excretion, and the uptake limitation by altering the filtration rate or tissue isolation from the external medium by valve closing (Almeida et al., 2017; Contardo-Jara et al., 2011b; Freitas et al., 2015; Garcia et al., 2012). The BCF values obtained after 21 day exposure were low (from 0.76 to 0.91 L kg^{-1}) indicating a low bioconcentration potential for FMQ, CIP and IBU in this organism (chemicals are considered bioaccumulative when their BCF is ≥5000 L kg^{-1}, de Solla et al., 2016). The BCF values were similar at both

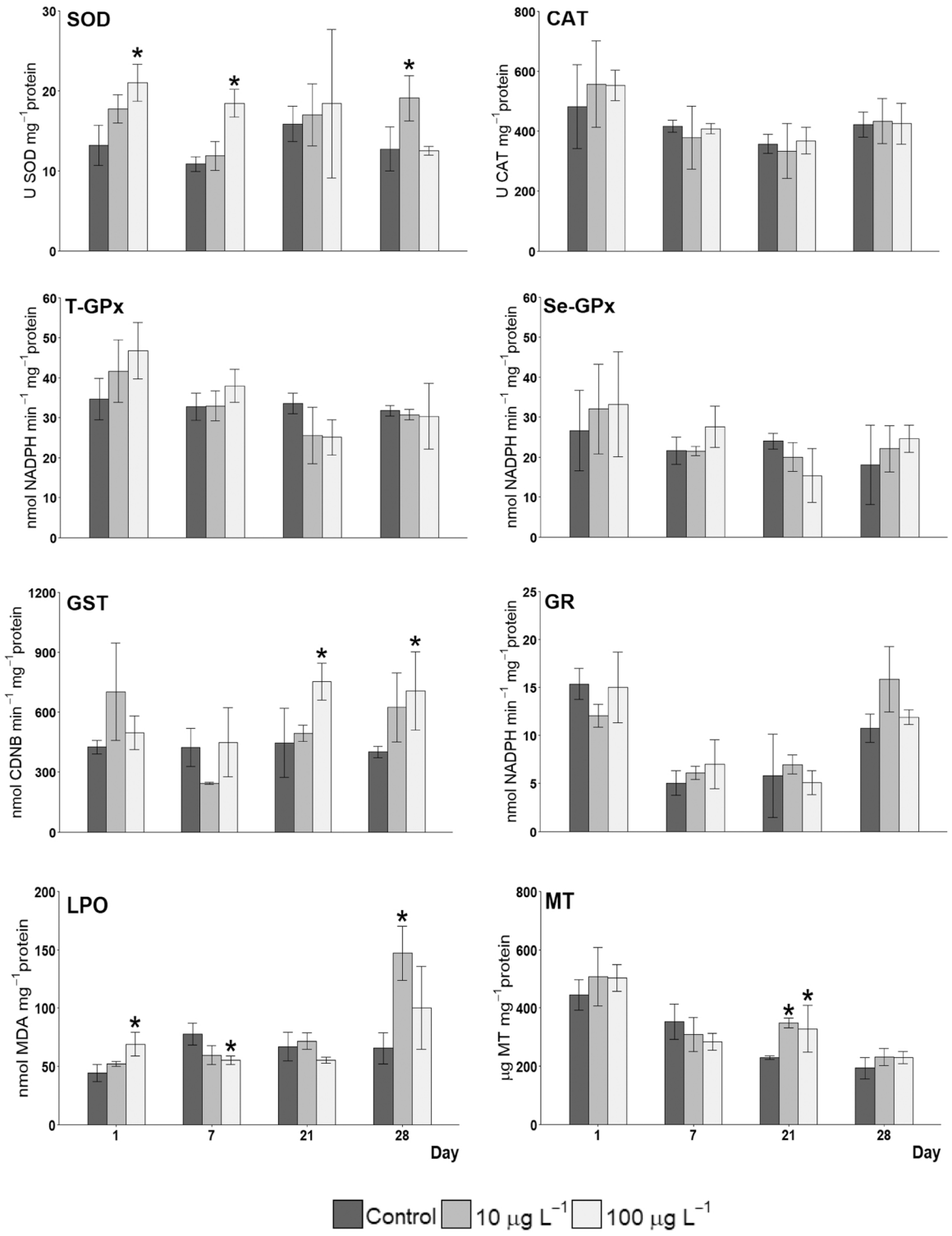

Fig. 1. Oxidative stress responses (superoxide dismutase SOD, catalase CAT, total-glutathione peroxidase T-GPx, Se-dependent glutathione peroxidase Se-GPx, glutathione S-transferase GST, glutathione reductase GR, lipid peroxidation LPO, metallothionein MT) measured in the digestive gland of *Scrobicularia plana* along the exposure (at 10 and 100 µg drugs L^{-1}, days 1, 7 and 21) and depuration (day 28) phases. Results are expressed as mean ± standard deviation (n = 3 replicates of pooled samples). Asterisks indicate significant differences (Tukey, $p < 0.05$) from the control.

exposure conditions indicating that an increase of mixture concentration does not affect uptake and elimination mechanisms of these drugs.

Additionally, the results (both the decrease in the drug content in the whole body and the detection of the three compounds in the aquatic medium) obtained for the depuration phase confirm that clams are able to progressively reduce the internal content of contaminants. In mammals, all three compounds are usually cleared fairly quickly from the body after administration of the last dose: for IBU, 90 % of the administered dose is eliminated after 24 h whereas the excretion of CIP and FMQ is slower, requiring 5–7 days for their total elimination (https://www.drugbank.ca, htpps://pubchem.ncbi.nlm.nih.gov). Removal of the three compounds from clam body appears not to be as fast. The set

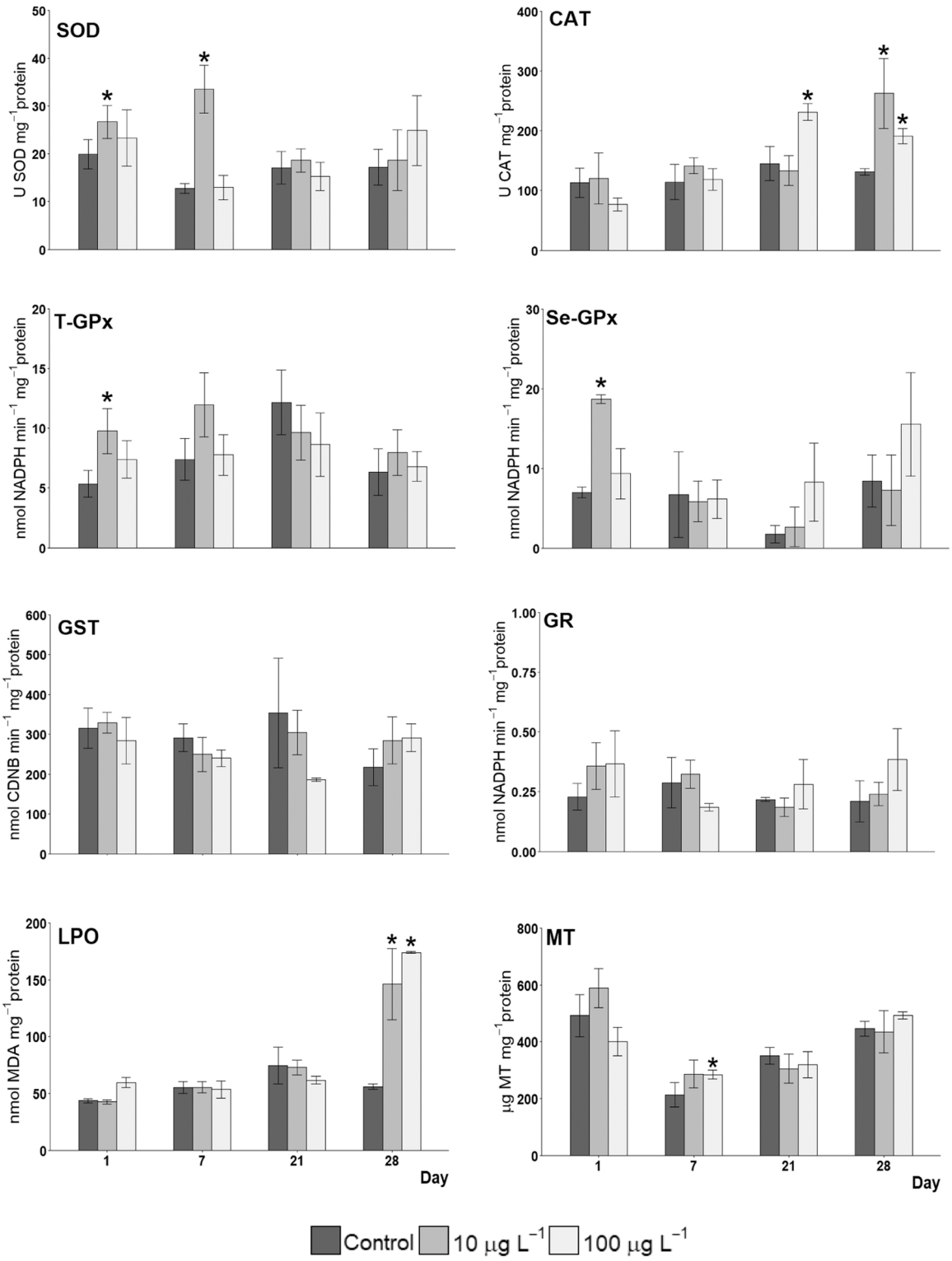

Fig. 2. Oxidative stress responses (superoxide dismutase SOD, catalase CAT, total-glutathione peroxidase T-GPx, Se-dependent glutathione peroxidase Se-GPx, glutathione S-transferase GST, glutathione reductase GR, lipid peroxidation LPO, metallothionein MT) measured in the gills of *Scrobicularia plana* along the exposure (at 10 and 100 µg drugs L^{-1}, days 1, 7 and 21) and depuration (day 28) phases. Results are expressed as mean ± standard deviation (n = 3 replicates of pooled samples). Asterisks indicate significant differences (Tukey, $p < 0.05$) from the control.

of enzymes responsible of the xenobiotic metabolism in mollusks is more restricted than in humans and consequently a less efficient pharmaceutical metabolization could be observed (Serra-Compte et al., 2019). Additionally, we can suppose that drug elimination can be slowed down by the selected exposure system: water renovation every 48 h makes excreted drugs persist in the exposure medium until the next water change, and can be taken up by organisms again. However, additional studies would be necessary to prove it.

An unexpected high mortality was observed throughout the experiment: however this was observed both in the controls and in the clams

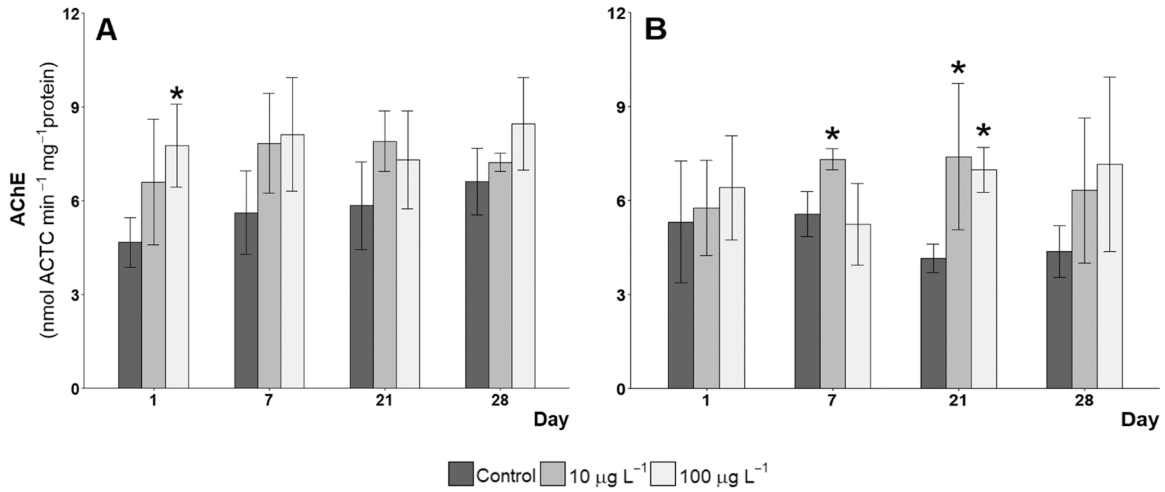

Fig. 3. Acetylcholinesterase activity (AChE) in digestive gland (A) and gills (B) of *Scrobicularia plana* specimens exposed to the pharmaceutical mixture at 10 and 100 μg L^{-1}. Values represent the mean ± standard deviation for n = 3 replicates of pooled samples (n = 3). Asterisks indicate significant differences (Tukey, $p < 0.05$) from the control.

exposed to the drug mixtures. Although a trend towards increased mortality was observed in organisms exposed to the drug mixture with respect to the control (not significant), we can speculate that the cause of the high mortality is not the presence of the pharmaceuticals in the exposure medium: perhaps the same experimental system chosen (semi-static conditions instead of open circuit with continuous water replacement, maybe poor nutrition, etc.) is a source of stress that contribute to enhance the clam mortality.

Many studies have shown the ability of pharmaceuticals to act as pro-oxidants also at very low doses (Fabbri and Franzellitti, 2016). The induction of responses associated with oxidative damage, antioxidant and detoxification mechanisms by IBU was observed in different bivalve species (*Corbicula fluminea*, *Dreissena polymorpha*, *Mytilus galloprovincialis* and *Ruditapes philippinarum,* Supplementary Material S6). However, the information regarding the toxicity of the selected antibiotics on aquatic organisms is very scarce. CIP has shown to induce an increase in hemocyte ROS content in *Elliptio complanata* and the modulation of oxidative stress responses in aquatic plants (*Ricciocarpus natans* and *Lemna minor*), microalgae (*Pseudokirchneriella subcapitata*), amphibians (*Rhinella arenarum*), crustaceans (*Daphnia magna*) and fish (*Oreochromis niloticus*) (Supplementary Material S6). In the case of FMQ, only data about acute toxicity in bacteria (*Vibrio fisheri* and cyanobacteria), crustaceans (*D. magna* and *Artemia* pp.), algae and aquatic plants (*P. subcapitata*, *L. minor* and *Lythrum salicaria*) were found in the existing bibliography (Supplementary Material S6) and no information was found about effects induced by sublethal doses.

The biochemical responses were altered throughout the exposure phase both in digestive gland and gills probably due to an imbalance in the redox state of exposed clams.

In digestive gland of organisms exposed to the highest dose of the mixture (100 μg L^{-1}) we recorded an increase in SOD activity, indicative of oxidative stress possibly associated with the rapid pharmaceutical accumulation. However this alteration was temporary and SOD levels were lower or similar to the control at the end of the exposure period. Alterations of other biomarkers related to the oxidative stress (MT, GST and LPO) indicate the activation of protection mechanisms against possible oxidative damage produced by drugs. GST is a key enzyme of the cellular detoxification system involved in the conjugation of reduced glutathione GSH with xenobiotics compounds and, additionally, in the inactivation of lipid peroxidation products (Regoli and Giuliani, 2014). MTs are also considered molecules with antioxidant role due to their ability to react with hydroxyl radicals (Amiard et al., 2006); this could explain the increase in MT levels observed in the digestive gland in this study. SOD acts as the first defense line against ROS, catalyzing the dismutation of superoxide radicals to H_2O_2 that is subsequently detoxified to oxygen and water by CAT. In the digestive gland, no changes were observed in CAT activity, which lead us to hypothesize that other mechanisms are responsible for the conversion of H_2O_2 to non-toxic forms; for example, GPx catalyzes the reaction $H_2O_2 \rightarrow H_2O + O_2$. In a previous study (Trombini et al., 2021) we observed that the same drug mixture (100 μg L^{-1}) produced alterations in the antioxidant system of the hepatopancreas in the crayfish *Procambarus clarkii*: an increase both in the activity and in the protein abundance for CAT, SOD and GST was observed proving the ability of the mixture of CIP, FMQ and IBU to modify the redox status in exposed organisms. In the present study we also recorded increased AChE activity throughout the entire exposure phase to the high mixture dose. Generally, neurotoxic effects of pollutants are associated with decreased AChE activity, however, several authors observed that exposure to pharmaceuticals produces the opposite effect in bivalve molluscs (Aguirre-Martínez et al., 2016, 2018; Fontes et al., 2018). Zhang et al. (2002) indicates that, in human and mammals, this may be due to an alteration in the normal functioning of the enzyme as a consequence of the increase in the intracellular ROS content (AChE release and induction after cell membrane disruption in apoptotic processes). An alternative explanation is recently given by Kim and Lee (2018) who, in a review of AChE functions in invertebrates (nematodes, arachnids and insects) indicated the existence of non-classical functions (no related with neurotransmission) for this enzyme; the authors observed that some soluble AChE forms, abundant in non-neuronal tissues, are induced by contaminants, suggesting their involvement in defence mechanisms against xenobiotics.

At low exposure dose, the drug mixture induces alterations related to oxidative stress, although to a lesser extent with respect to higher doses. Despite not observing significant differences between control and exposed organisms (with exception of MT), the graphs representing the temporal evolution of biomarkers show a trend towards higher (SOD, T- and Se-GPx and AChE at day 1, and AChE at day 7 and 21) or lower (GST and LPO at day 7, T- and Se-GPx at day 21) average values with respect to the control. Low-dose drug mixture could induce an increase in the ROS cellular content associated to the processes of pharmaceuticals metabolization and elimination; in this case it would be a less important increase and small changes in the antioxidant system would be able to regulate the ROS cellular content, avoiding oxidative stress and the associated damage. Mezzelani et al. (2016) observed no changes in the antioxidant system of *M. galloprovincialis* (digestive gland) exposed to a low dose of IBU (25 μg L^{-1}) during 15 days, arguing that this compound

exerts a limited oxidative effect on the mollusc. Similar results were obtained by Gonzalez-Rey and Bebianno (2011) in the same organism exposed to an even lower concentration of IBU during 15 days.

The gills were also affected by the exposure to the drug cocktail at both doses. For organisms exposed to 100 μg L^{-1} a general alteration of the redox status was observed. Alteration induced by the low exposure dose appears to be greater than at the high dose, particularly during the first week of the experiment in which an increase in SOD (days 1 and 7) and AChE (day 7) activity was observed. In the case of AChE, the activity remained higher until the end of the exposure phase. Based on our results, we can suggest that, at both exposure concentrations, defence mechanisms against the redox imbalance potentially induced by the drug mixture are activated in gill and no oxidative damage is produced (LPO levels did not increase throughout the entire exposure). However, it seems that different mechanisms are activated at different exposure doses. In the case of the lowest dose, we observed an antioxidant defence pathway similar to the digestive gland in which SOD catalyzes the conversion of $O_2^{\cdot}$ to H_2O_2 and other molecules (maybe GPx whose levels showed an increase after 1 day of exposure) the transformation of the latter into H_2O. The activation of enzymes in the early phase of exposure indicates that, at low drug concentration, gills (the first organ to come into contact with the contaminants present in the aquatic environment and therefore more sensitive than digestive gland) react quickly to prevent the oxidative stress and the derived damages. In the case of exposure to the highest pharmaceutical dose, alterations were observed in CAT and MT after 21 and 7 days of exposure respectively, although the statistical tests do not relate them to the presence of drugs in the exposure medium. Contardo-Jara et al., (2011a) observed an over-expression of genes encoding for MT and CAT in gills of *M. galloprovincialis* specimens exposed to 206.3 μg IBU L^{-1} for 4 days. Several authors observed that IBU induces the inhibition of various enzymes (SOD, CAT, GST, GPx) in bivalve species (Aguirre-Martínez et al., 2016). Pharmaceutical exposure, particularly at high concentrations, could therefore alter normal cellular processes by inhibiting the activity of some enzymes and inducing the activation of alternative protection mechanisms such as the increase in MT content. At both exposure levels we observed an increase in AChE activity that, as in the digestive gland, could be interpreted, on the one hand, by the involvement of AChE in the protection processes against xenobiotic compounds and, on the other, by the alteration of the normal function of this enzyme (neurotoxic effect) as a consequence of the exposure to the pharmaceutical mixture.

The results obtained for biochemical parameters after the depuration phase indicate that both tissues present a stress condition, even higher than that observed at the end of exposure phase (with exception of digestive gland of organisms exposed to the mixture at 100 μg L^{-1}). The increase in LPO levels (gills and digestive gland), CAT (gills, both doses), SOD and GST (digestive gland,10 and 100 μg L^{-1} respectively) activities indicate that organisms are still subject to oxidative stress that in this phase causes damage to lipids. In this case the stress could be induced by the presence of the drugs and their metabolites in the exposure medium. The pharmaceuticals are metabolized by organisms and excreted as a mixture of parent compounds and various metabolites depending on type of compound. In the case of humans, IBU is almost totally metabolized and only a small percentage ($\leq$5 %) is excreted in unaltered form, however CIP and FMQ are mostly eliminated ($\geq$70 %) as parent compounds. Aquatic invertebrates are able to metabolize pharmaceutical compounds in a similar way to humans (Bonnefille et al., 2017) therefore clams are probably exposed to a mixture of IBU, CIP, FMQ and their metabolites throughout the depuration phase. The metabolites may have significant activity and hence toxic effects (in some cases even greater than their parent compounds), on exposed organisms (Jjemba, 2006).

Additionally, the increased stress level and the oxidative damage observed during the depuration phase can also be justified by the hypothesis that during exposure IBU has a double function acting on the one hand as a pro-oxidant together with the other pharmaceuticals present in the mixture and, on the other, as an anti-inflammatory by its pharmacological mechanism reducing the oxidative effects in the tissues of *S. plana* (Leone et al., 2007). The joint action of IBU and antioxidant defence mechanisms triggered in the clams could reduce the stress status and therefore explain the absence of lipid membrane damage observed during the exposure phase, damage that actually appears during the depuration phase when drug administration stops.

5. Conclusions

This study aimed to investigate the toxicological potential of the mixture of ibuprofen, ciprofloxacin and flumequine in *S. plana*. The results showed that clams bioaccumulate the three compounds in a similar way, being able both to maintain their levels constant throughout a prolonged exposure and to reduce their internal content in the depuration phase. Additionally our data indicated that the presence of pharmaceutical compounds in the aquatic medium induces biochemical alterations in target tissues. We focused on responses related to oxidative stress, detoxification processes and neurotoxicity and particularly on their temporal evolution, an approach to understand the defence and recovery mechanisms of organisms. The redox pathways of both target tissues were altered by exposure to the drug mixture but in different ways depending on time and exposure dose indicating different defence strategies and sensitivity. Finally, the evaluation of selected responses after a depuration period can be useful to test the recovery capability of organisms and detect the induction of permanent damage. Our results showed signs of oxidative damage (increased levels of LPO) after this phase although further studies are needed to clarify this issue.

CRediT authorship contribution statement

Chiara Trombini: Formal analysis, Investigation, Writing – original draft, Visualization. **Julia Kazakova:** Formal analysis, Investigation, Writing – original draft. **Mercedes Villar-Navarro:** Investigation, Writing – review & editing. **Miriam Hampel:** Writing – review & editing, Supervision. **Rut Fernández-Torres:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Miguel Ángel Bello-López:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Julián Blasco:** Conceptualization, Funding acquisition, 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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2022.113845.

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