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Assessment of pharmaceutical mixture (ibuprofen, ciprofloxacin and flumequine) effects to the crayfish *Procambarus clarkii*: A multilevel analysis (biochemical, transcriptional and proteomic approaches)

Chiara Trombini [a,*], Julia Kazakova [b], Alejandro Montilla-López [c], Ricardo Fernández-Cisnal [c], Miriam Hampel [d], Rut Fernández-Torres [b], Miguel Ángel Bello-López [b], Nieves Abril [c], Julián Blasco [a]

[a] Department of Ecology and Coastal Management, Instituto de Ciencias Marinas de Andalucía (CSIC), Campus Rio San Pedro, 11510, Puerto Real, Cádiz, Spain
[b] Department of Analytical Chemistry, Faculty of Chemistry, Universidad de Sevilla, 41012, Spain
[c] Department of Biochemistry and Molecular Biology, Universidad de Córdoba, Campus Universitario de Rabanales, 14071, Córdoba, Spain
[d] Instituto Universitario de Investigación Marina (INMAR), Campus Rio San Pedro, 11510, Puerto Real, Cádiz, Spain

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ABSTRACT

The knowledge about the effects of pharmaceuticals on aquatic organisms has been increasing in the last decade. However, due to the variety of compounds presents in the aquatic medium, exposure scenarios and exposed organisms, there are still many gaps in the knowledge on how mixtures of such bioactive compounds affect exposed non target organisms. The crayfish *Procambarus clarkii* was used to analyze the toxicity effects of mixtures of ciprofloxacin, flumequine and ibuprofen at low and high concentrations (10 and 100 μg/L) over 21 days of exposure and to assess the recovery capacity of the organism after a depuration phase following exposure during additional 7 days in clean water. The crayfish accumulated the three compounds throughout the entire exposure in the hepatopancreas. The exposure to the mixture altered the abundance of proteins associated with different cells functions such as biotransformation and detoxification processes (i.e. catalase and glutathione transferase), carbohydrate metabolism and immune responses. Additionally changes in expression of genes encoding antioxidant enzymes and in activity of the corresponding enzymes (i.e. superoxide dismutase, glutathione peroxidase and glutathione transferase) were reported. Alterations at different levels of biological organization did not run in parallel under all circumstances and can be related to changes in the redox status of the target tissue. No differences were observed between control and exposed organisms for most of selected endpoints after a week of depuration, indicating that exposure to the drug mixture did not produce permanent damage in the hepatopancreas of *P. clarkii*.

1. Introduction

A large variety of pharmaceuticals (275 to date, Mezzelani et al., 2018) are present worldwide in the aqueous environment, including influents and effluents of wastewater treatment plants WWTPs, surface (rivers, lakes and coastal waters), ground and drinking waters. Medications from human and veterinary use can enter the aquatic environment through different routes but effluents from sewage treatment plants (STPs) are generally recognised as the main source of these substances (Daughton and Ternes, 1999; Fent et al., 2006; Heberer, 2002; Yang et al., 2017). After consumption/application drugs reach, largely in unaltered form, the STPs where conventional processes are not effective in their removal or degradation. Therefore, a large number of pharmaceuticals are present in effluents from STPs and may reach different aquatic compartments (Behera et al., 2011; Castiglioni et al., 2006; Nakada et al., 2006; Paxéus, 2004; Santos et al., 2007). The continuous release from multiple pathways combined with the low degradation rate in the aqueous medium due to their chemical

* Corresponding author.
E-mail addresses: chiara.trombini@icman.csic.es (C. Trombini), jukaz1@alum.us.es (J. Kazakova), alejandro.montilla@ehu.eus (A. Montilla-López), g82fecir@uco.es (R. Fernández-Cisnal), miriam.hampel@uca.es (M. Hampel), rutft@us.es (R. Fernández-Torres), mabello@us.es (M.Á. Bello-López), bb1abdim@uco.es (N. Abril), julian.blasco@csic.es (J. Blasco).

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properties makes pharmaceuticals "pseudo-persistent" contaminants (Bu et al., 2016; Ebele et al., 2017; Richman and Castensson, 2008). Consequently, aquatic organisms are continually exposed to a complex mixtures of pharmaceuticals and, although levels of these compounds in the aquatic environments are generally very low ranging from ng/L to low μg/L, pharmaceuticals are highly reactive molecules designed to interact with biological systems even at low concentrations and hence sub-lethal, long-term effects can be expected (Bu et al., 2013; Desbiolles et al., 2018; Petrie et al., 2015; Ying et al., 2013). Due to the potential hazard of these compounds, research into pharmaceutical pollution has extensively expanded in the last decade (Kay et al., 2017). Even so, most works have focused on the global analysis of drugs in the aquatic environment (detection and quantification in different aquatic compartments, sources, removal efficiency in STPs, risk assessment, etc.) and toxic effects of pharmaceuticals as single compounds. Studies focusing on the effects of pharmaceutical mixtures on aquatic organisms are thus necessary, particularly under chronic exposure conditions (David et al., 2020; Hossain et al., 2021).

This work centres on three pharmaceuticals commonly detected in the freshwater aquatic compartment. Ibuprofen (IBU), one of the most used non-steroidal anti-inflammatory drugs (NSAID), is commonly detected in surface waters with concentrations ranging from <0.3 ng/L to 16 μg/L (Valcarcel et al., 2011; Ying et al., 2013; Ziylan and Ince, 2011). This compound has been detected in different fish and invertebrate tissues and has shown to induce sublethal adverse effects (behavioural changes, immunological and haematological alterations, modulation of enzyme activities and gene expression, endocrine disruption, genotoxic effects, etc.) both in fresh and saltwater organisms (Gonzalez-Rey and Bebianno, 2011; Maranho et al., 2014; Mathias et al., 2018; Mezzelani et al., 2018; Miller et al., 2018; Parolini et al., 2011; Serrano et al., 2015; Xia et al., 2017). Ciprofloxacin (CIP) and flumequine (FLU) belong to the broad-spectrum antibiotics fluoroquinolones, a class of special environmental concern due to their extensive use and the high resistance to abiotic and biotic degradation (Ebert et al., 2011; Robinson et al., 2005a). Liu et al. (2018) include CIP among the most frequent antibiotics detected in aquatic environments; concentrations of this compound ranging from ng/L to μg/L (0.1–9660 ng/L) have been reported in different water resources worldwide (Carvalho and Santos, 2016; Bradley et al., 2017) and acute and chronic toxicity (particularly at concentrations > mg/L) was studied in a variety of aquatic groups particularly algae, plants and bacteria revealing the induction of deleterious effects (mortality, growth and reproduction inhibition, photosynthesis efficiency decrease, etc.) in exposed organisms (Ebert et al., 2011; Gomes et al. 2017, 2018, 2018; Halling-Sorensen et al., 2000; Martins et al., 2012). Nevertheless very few works have been conducted to study the CIP effects on more complex aquatic organisms (invertebrates and vertebrates) and, even less, at environmentally relevant concentrations and focusing on molecular and submolecular endpoints (Gust et al. 2012, 2013, 2013; Halling-Sorensen et al., 2000; Martins et al., 2012; Maul et al., 2006). Data available for FLU, the second antimicrobial selected in this study, are even scarcer than for CIP and only limited to its detection in aquatic environments (10–41 ng/L, Carvalho and Santos, 2016) and its acute toxic effects on a few species of algae, bacteria and crustaceans (Brian et al., 2007; Migliore et al., 1997; Robinson et al., 2005).

Crustaceans have been widely used in ecotoxicological research (Lyubenova and Boteva, 2016). The crayfish *Procambarus clarkii* (Girard, 1852), native of the southern United States, was introduced worldwide assuming an important role in the trophic chain of colonized systems and, nowadays is one of the most common crayfish species cultured commercially for human consumption (Gonçalves Loureiro et al., 2015; Liu et al., 2011; Moss et al., 2010; Sheir et al., 2015). Due to the high knowledge of its biological characteristics and its ecologic and economic importance, *P. clarkii* has been widely used as a model species for the assessment of contaminant effects in field and are considered as a good model organism to study changes induced by pollutants (Osuna-Jiménez et al., 2014; Serrano et al., 2000; Suárez-Serrano et al., 2010; Vioque-Fernández et al., 2009a; Yu et al., 2018). The use of a species as a bioindicator needs preliminary studies to characterize and understand the responses to environmental stressors, and this knowledge is critical for interpreting changes in biological responses and using them as an early warning signal of pollutant detection. Information about the adverse effects of pharmaceuticals on *P. clarkii* is very scarce. This study focused on the mixture of selected compounds (a more environmentally realistic exposure scenario, since in aquatic systems organisms are simultaneously exposed to many chemicals) and we tested the hypothesis that, at environmentally relevant dose, it is able to induce sub-lethal responses (particularly responses related with oxidative stress/damage or with depuration processes) in target tissues of *P. clarkii*. Based on the idea that contaminants interact at different biological organization levels (molecular, cellular, tissue, etc.) and that alterations can be related to each other (cascade of events from molecular to higher organization levels for example) (Ankley et al., 2010) we choose a multivariate approach integrating the study of conventional toxicological biomarkers, expression of selected genes and the massive analysis of effects at the proteomic level. We also suppose that different factors can modulate all this responses between them chemical dosage, exposure duration (short, medium or long-term) and intermittent exposure (exposure and depuration phases).

Animals were exposed to the drug mixture for 21 days at two sublethal doses, the first one (10 μg/L) closest to environmentally relevant concentrations and other ten fold higher (100 μg/L), similar to levels detectable in highly contaminated areas or receiving waters from some specific locales such as hospitals, pharmaceutical factories, farms, etc. (Chopra and Kumar 2020; Carvalho and Santos, 2016) and supposedly capable of inducing distinguishable responses in the organisms, to determine the effects of the mixture in the hepatopancreas, the main organ involved in nutrient storage and a variety of immune responses and detoxification processes (Gibson, 1979). Biochemical parameters (oxidative stress, neurotoxicity and detoxification biomarkers), gene transcription and protein expression were evaluated at different times (after 1, 7 and 21 days) to assess their evolution in the short and medium term and try to understand the possible toxicity mechanisms of the mixture. Additionally, pre-exposed organisms were depurated in clean freshwater for 7 days and bioaccumulation, biochemical biomarkers and gene expression were evaluated in the *P. clarkii* hepatopancreas with the aim of investigating the recovery capability of the organisms (if the possible toxic effects are permanent or reversible) after removal of pharmaceuticals from the exposure medium.

2. Materials and methods

2.1. Animals, experimental procedure and sampling

Specimens of *P. clarkii* were collected at the lagoon of "La Janda" (36°13′05.6″N, 5°47′02.3″W, Cádiz, Spain). Before the beginning of the experiment, crayfish were acclimated in aerated, dechlorinated freshwater to laboratory conditions forat least1 week. Exposure was carried out for 21 days in tanks with aerated and dechlorinated freshwater (1L/animal) under natural photoperiods. Plastic tubes were placed in the tanks to avoid cannibalism. Pharmaceuticals, obtained from Sigma Aldrich (Steinheim, Germany, purity≥98%, main characteristics shown in Supplementary material, Table S1), were added to the exposure tanks as a stock solution prepared in dimethyl sulfoxide (final DMSO concentration in the tanks: 0.002% v/v) to reach the final nominal concentration of 10 and 100 μg/L (concentration of each compound present in the mixture) and every condition, DMSO control (control), mixture at 10 μg/Land mixture at 100 μg/L, was tested in triplicate. Water was completely changed and chemical renewed every 48 h and organisms were fed with extruded sinking fish feed pellets (Skretting, Nutreco Company, Spain) 3 h before the water change.

Biological sampling was carried out at the start of the experiment

(day 0, from the acclimatization tank) and after 1, 7 and 21 days. Every sampling day, organisms were chosen randomly (14 animals per tank) and the hepatopancreas was excised, frozen in liquid nitrogen and stored at −80 °C until further analyses. Tanks were cleaned and the remaining animals were maintained for 7 additionally days to assess their possible recovery in depuration conditions. Water change and feeding intervals were performed as during the exposure phase.

Water sampling was regularly performed to quantify pharmaceutical concentrations and ammonium content in the tanks.

Temperature, pH, conductivity and dissolved oxygen were recorded routinely in the exposure medium (17.8±1.6 °C, 8.3±0.2, 477.4±30.5 μS/cm and 8.5±0.8 mg/L respectively) both during exposure and depuration phases and crayfish mortality daily checked (2.5% average mortality).

2.2. Chemical analysis

Hepatopancreas samples (15 organisms per condition: 5 individual hepatopancreas × 3 tanks) were individually lyophilized (Labconco Freezone 2.3, MA, USA) and then extracted by applying microwave energy (5 min, 50 W power, Ethos 900, Milestone)). Lyophilized sample (0.1 g) was treated with 2 mL of a mixture acetonitrile (ACN): water 1:1 (v/v) spiked with 10 μL proteinase-K PCR recombinant of 20.2 mg/mL (Roche Diagnosis, Barcelona, Spain) and 1 μL of formic acid. The obtained extracts were then centrifuged (Hettich Universal 320) for 10 min at 9000 rpm, and evaporated under nitrogen 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 (XEVO G2-S Waters Corporation, Mildford, MA, USA) injection.

Water samples were microfiltered (0.2 μm) and a 10 μL aliquot was directly injected into LC-ESI-QTOF-MS system.

The chromatographic separation was performed on an Acquity UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm, Waters Corporation, Milford, MA, USA). The mobile phase was water (A) and ACN (0.1% formic acid) (B). A gradient elution program was applied for 10 min consisting in 90% A initially to a 45% A in 4.5 min, a subsequent isocratic step for 3.5 min returning to initial conditions at t= 8.0 min. 2 min were waited for reequilibration before the following injection. The flow rate was set at 0.3 mL/min. Detection was carried out at Xevo G2S QTOF mass spectrometer, in full scan resolution mode and ESI positive/negative mode was applied for ionization of the analytes.CIP, FLU and IBU analytical standards (from Sigma-Aldrich, Merck Life Science S.L.U., Spain) were used for standard curves. Instrumental limits of quantification (LOQs) resulting for the method were in the range of 3.12–4.19 ng/g for biological sample analysis (CIP: 3.21, FLU: 4.19, IBU: 3.12) and 1.92–2.73 ng/L for water sample analysis (CIP: 1.92, FLU: 2.73, IBU: 2.12). Instrumental limits of detection (LODs) were in the range of 0.99–1.99 ng/g (CIP: 0.99, FLU: 1.65, IBU: 1.99) and 0.16–0.94 ng/L (CIP: 0.16, FLU: 0.89, IBU: 0.94) respectively for biological sample and water samples analysis.

Ammonium levels in water samples were quantified according to the method of Krom (1980) and varied from 1.0 to 9.3 μmol/L.

2.3. Biochemical analysis

Hepatopancreas samples (6 organisms per condition: 2 pooled hepatopancreas x 3 tanks) were homogenized (weight: volume ratio of 1:4) with 50 mM Tris buffer (150 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% antiproteolitic cocktail) pH 7.4 using an Ultra-turrax homogenizer (ULTRA-TURRAX T25, Janke&Kunkel, IKA®Labortechnich). The homogenate was then centrifuged at 12000×*g* at 4 °C during 30 min to obtain a supernatant (S12 fraction) and aliquots were collected for biochemical analysis.

2.3.1. Total protein, enzyme activity analysis and lipid peroxidation

All biochemical determinations were performed in triplicate by spectrophotometry (microplate reader Tecane Infinite 200, Tecane Group Ltd, Austria).

Total protein content was determined according to Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as standard and was used for normalization of data for biomarkers.

Catalase (CAT) activity was evaluated measuring the absorbance decrease (230 nm) due to the hydrogen peroxide consumption during 5 min (30 s intervals) according to the method described by Beers and Sizer (1952) and modified by Li and Schellhorn (2007). CAT activity was expressed as U CAT/mg proteins where one unit of enzyme (U) is defined as the amount of enzyme that caused the loss of 1.0 μmol of H_2O_2 per min, under the assay conditions.

Superoxide dismutase (SOD) activity was assessed using a SOD Assay Kit-WST (Dojindo Laboratories, Japan) according to the method of Ukeda et al. (1999) based on the reaction of water-soluble tetrazolium salt (2-(4-iodophenyl)-3-(4—nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt, WST-1). SOD from bovine erythrocytes was used as standard. Results were expressed as U SOD/mg proteins where unit of SOD (U) is defined as the amount of the enzyme in the sample volume that inhibits the reduction reaction of WST-1 with the superoxide anion by 50%.

Glutathione reductase (GR) determination was conducted following the method of Cohen and Duvel (1988) modified for microplate reader by McFarland (1999). The loss of NADPH present in the reaction mixture was recorded at 340 nm over 10 min (1 min intervals) and activity was reported as nmol NADPH consumed/min/mg proteins.

Glutathione-S-transferase (GST) activity was determined following the method described by McFarland (1999) using 1-chloro-4,4-dinitrobenzene as a substrate. Absorbance was recorded at 340 nm during 3 min (30 s intervals) and activity was reported as μmol CDNB/min/mg proteins.

Total glutathione transferase (T-GPx) assay was conducted using cumene hydroperoxide as substrate (McFarland, 1999) and the loss of NADPH present in the reaction mixture was monitored as the reaction progress (absorbance at 340 nm during 3 min, 10 s intervals). Activity was expressed as nmol NADPH/min/mg proteins.

Lipid peroxidation (LPO) levels were quantified following the thiobarbituric acid reactive substances (TBARS) method as described by Hannam et al. (2010). Oxidative stress leads to malondialdehyde (MDA) production as final product of oxidative lipid degradation (Janero, 1990). The 1,1,3,3-tertramethoxypropane produced in the reaction between MDA and trichloroacetic acid can be quantified spectrophotometrically (535 nm) as indirect measure of MDA. Results were expressed as nmol MDA/mg proteins.

Acetylcholinesterase (AChE) activity was determined using the method described by Matozzo et al. (2012) in which reaction progress between S-acetylthiocholine iodide (ACTC) and dithiobisnitrobenzoate is followed by recording absorbance (405 nm) changes during 5 min (30 s intervals). Activity was expressed as μmol ACTC/min/mg proteins.

2.3.2. Metallothionein

Total metallothionein (MT) content was determined in the supernatant by reverse phase high performance liquid chromatography (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, 2011) and Romero-Ruiz et al. (2008). Supernatant was derivatized with the fluorogenic reagent monobromobimane (mBBr) after treatment at 70 °C with ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (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 proteins.

2.4. Gene expression

For gene expression analysis, total RNA was extracted from *P. clarkii* hepatopancreas (30 mg from a pulverized with nitrogen pool, 2 organisms × 3 tanks per condition) using the NucleoSpin® RNA isolation kit. RNA concentrations and quality were verifiedusing a Bioanalyzer2100 (Agilent) and a NanoDrop 2000c (Thermo Scientific)spectrophotometer. The cDNA synthesis was performed using iScript™ cDNA synthesis kit.

Specific primers (*gadph*, *b-act*, *gpx*, *cat*, *Mnsod*, *gst*, *mt*, *Colα*, *SnAp* and *Thy*, Supplementary Table S2) for real-time PCR (q-PCR) were designed from RNA sequences obtained from GenBank (National Center for Biotechnology Information, NCBI) using the software Primer3 Input (version 0.4.0) and oligonucleotides were obtained from Biomers® (Ulm, Germany).

The qPCR amplification was conducted according to the manfacturer's recommended procedure (Fluorescent Quantitative Detection System Mastercycler ep *realplex*^{2} S, Eppendorf) using PerfeCta®SYBR® Green FastMix®as the detection chemistry. Parameters for q-PCR were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s (amplification), annealing at the optimal temperature (Supplementary Table S2) for 30 s and elongation at 72 °C for 30 s. All reactions were performed in triplicates and a melting curve was generated at the end of the run to confirm the specificity of the amplification products. The relative expression levels of the genes was normalized using *b-act* and *gadph* as reference genes by the geNorm software (VBA application for Microsoft excel) (Vandesompele et al., 2002).

2.5. 1DE-proteomic analysis

The fifteen crayfish included in each experimental condition (15 organisms per condition: 5 organisms × 3 tanks) were randomly distributed in three subgroups. Equal amounts of cryo-homogenized hepatopancreas tissue from each subgroup were pooled and used to determine, in triplicate, the differences in protein abundance caused by the treatment. Hepatopancreas tissue (100 mg) from each pool was homogenized in 300 μL of Tris-HCl 20 mM, pH 7.6, containing 0,5 M sucrose; 0,15M KCl; 20 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 6 μM leupeptin and protease inhibitors (Sigma Protease Inhibitor P2714). Cellular debris was removed by three consecutive centrifugations (14,000×*g*, 10 min, 4 °C). Benzonase (500 U/mL) was added to each protein sample and the mixtures incubated for 30 min to eliminate nucleic acids. Samples were then centrifugated (100,000×*g*, 60 min, 4 °C, OptimaTM TLX Ultracentrifuge, Beckman, rotor TLA-120.2) to precipitate the remaining non-peptide material. Protein concentration was quantified using the Bradford assay.

Isolated proteins were 1:1 mixed with 2X Laemmli SDS-PAGE buffer (BioRad), loaded (80 μg/lane) in triplicate onto 12% w/v SDS-PAGE gels (18 cm length) and separated by 1-DE gel electrophoresis (8W per gel for 1.5 h followed by 15 W per gel for for 5.5 h, 20 °C in aPROTEAN II XI Cell, Bio-Rad). Then, the gels were stained with SYPRO® Ruby Protein Gel Stain (Thermo Fisher) for 18 h in the dark and imaged in an FX Molecular Imager (BioRad) (532 nm excitation and 555 nm emission/detection). The Band Analysis tools of ImageLab software *v.* 4.1 (Bio-Rad) were used to select and determine the background-subtracted density of the bands. Fluorescent images of all the 1-DE gels were captured with the same sensitivity, and processed under the same parameter values to attain accurate inter-sample comparisons.

Bands with differential intensity among the reference and exposed crayfish samples were automatically excised in a ProPic station (Digilab Genomic Solutions). Gel slices were twice bleached (30 min, 37 °C) with 200 mM ammonium bicarbonate (NH_4HCO_3)/40% ACN and subjected to three additional washing steps with 100% ACN and 25 mM NH_4HCO_3/50% ACN. Finally, they were dehydrated with 100% ACN (5 min) and dried (4 h, room temperature, RT).

For protein identification, the protein samples were tryptically digested using an Investigator ProGest Digestion Station (Genomic solutions). The resulting peptides were extracted from the matrix (10 μL 10% trifluoroacetic acid TFA, 15 min, RT) and purified with C18 ZipTip (Millipore) microcolumns (Pro PepII station, Digilab Genomic Solutions Inc.). Eluents were directly loaded onto a MALDI plate using 27 mM α-cyano-4-hydroxycinnamic acid in 70% ACN and 0.1% TFA as the matrix solution. MALDI-TOF spectra were taken on AB SCIEX 4800 MALDI TOF/TOF™ Analyzer (Applied Biosystems) in automatic mode with an m/z range of 800–4000, at a 20-kV accelerating voltage. The MS spectra were internally calibrated using peptides obtained through trypsin autolysis (MH^+, 842.509 and 2211.104) and resulted in mass errors of less than 30 ppm. Filtered precursor ions were selected for the MS/MS scan.

Proteins were assigned identification by Peptide Mass Fingerprinting and MS/MS analysis. Mascot 2.0 search engine (Mascot Search Engine, Matrix Science) was used for protein identification running on GPS software v. 3.5 (Applied Biosystems) over UniProt Arthropods database (892576 sequences) in automated mode, establishing the following searching parameters: maximum allowed error of peptide mass of 100 ppm; cysteine as an Scarbamidomethyl-derivative; and oxidation of methionine. Non-identified profiles were then searched against the NCBI nr database (10,997,816 sequences) without any taxonomic restriction.

3. Data analysis

Statistical analyses were conducted using the statistical packages R (R Core Team, 2017) and InStat (version 3.05, GraphPad). Statistical significance was established at $p < 0.05$.

For bioaccumulation, biochemical and gene expression data one and two way ANOVA (after verification of data normality and homogeneity of variance by the Shapiro-Wilk and Levene test respectively) were used to study the effects of treatments and multiples comparisons (Tukey and T-test) were carried out to evaluate significant differences between treatments.

Statistically significant differences in protein abundance between each different dose and time and the reference sample was determined by using a Dunnet test with the Bonferroni correction, after assessing that the samples passed the Kolmogorov and Smirnov and Bartlett normality tests.

Data for pharmaceutical accumulation in the hepatopancreas were used to calculate the bioconcentration factor (BCF) as ratio between the analyte concentration in the tissue and in the exposure water (Arnot and Gobas, 2006).

The integrated biomarker response index (IBR) was used to obtain an integrated view of the changes produced by pharmaceutical mixtures on biochemical responses. IBR values for biochemical biomarkers and gene expression were calculated at different time intervals according to the method proposed by Beliaeff and Burgeot (2002) and modified by Devin et al. (2014). The calculations wereperformed using Excel and R software (Trombini et al., 2016).

PCA was applied (PCA, XLSTAT v. 2015.2.02, Addinsoft) to biomarkers, gene expression and proteomic data to evaluate the association between the different variables and the variance of each time set in exposed and unexposed organisms.

4. Results

4.1. Pharmaceuticals in exposure water and hepatopancreas

Test solutions were renewed every 48 h and were analyzed at regular intervals to compare the nominal versus measured concentrations. The concentrations of FLU, CIP and IBU measured during the exposure phase after addition of stock solution were in good agreement with nominal concentrations (Supplementary Table S3). Measured concentrations of pharmaceuticals decreased between 12 and 30% after 48 h renewal of

test solutions. All pharmaceuticals were detected in the exposure medium also during the depuration phase (water samples collected 72 h after starting depuration, Supplementary Table S3) indicating the existence of excretion and depuration processes that occur in the organisms during this phase. Pharmaceuticals were not detected in control water samples.

The bioaccumulation of drugs in the hepatopancreas along the entire experiment is showed in Fig. 1 (data from pharmaceutical quantification are showed in Supplementary Table S4). No pharmaceuticals were detected both in crayfish from acclimatization tanks and control organisms. The BCF values were reported for each compound and concentration at the end of the exposure phase (day 21, BCF_{21}). A progressive accumulation of all pharmaceuticals during exposure phase (Fig. 1) at both exposure levels was observed.

In crayfish exposed to the mixture at 10 μg/L, levels of FLU, CIP and IBU in the hepatopancreas at the end of exposure were approximately twice as much as recorded at day 1 and FLU was the most accumulated compound (BCF_{21}=2.33 L/kg for FLU versus 1.44 and 1.47 L/kg for CIP and IBU respectively, Fig. 1). After the depuration phase a decrease in the level of accumulated drugs was observed and the variation was dependent on the compound (41, 20 and 31% for FLU, CIP and IBU respectively). In the case of organisms exposed to the highest concentration (100 μg/L) tissue accumulation was similar for all pharmaceuticals as indicated by similar BCF_{21} values (1.77, 1.66 and 178 L/kg respectively for FLU, CIP and IBU, Fig. 1). After 7 days of depuration a considerable decrease of bioaccumulated drugs compared to the end of exposure phase was observed (47, 48 and 50% respectively for FLU, CIP and IBU).

4.2. Biochemical responses

Pharmaceuticals altered the biochemical biomarkers in the hepatopancreas. Exposure to the mixture at 10 μg/L induced significant increase ($p < 0.05$) of SOD activity after 1 and 21 days of exposure (Fig. 2) compared to the control while no significant changes were observed for the rest of biomarkers. More changes were observed in hepatopancreas of organisms exposed to the highest dose of pharmaceutical mixture (Fig. 2) with significant ($p < 0.05$) increase of SOD (day 21), CAT (day 7), T-GPx (day 7), and GST (days 7 and 21) activities and decrease of GR activity and LPO level (day 21). This is also confirmed by PCA results (Fig. 4A) concerning the exposure period: SOD, GST, T-GPx and LPO have strong correlation (0.79, 0.98, 0.81 and −0.78 respectively) with the first dimension F1 (that explains 47.18% of the total variance) and are positively associated (except LPO that shows negative association) with the lowest mixture concentration at day 7 and with both treatments at day 21. PCA dimension 2 explains18.59% of the total variance and strong correlation was observed with GR (0.79), negatively associated with the highest mixture concentration at day 21. After 7 days of depuration, enzyme activities and LPO levels in organisms pre-exposed to the pharmaceutical mixtures were similar to the control with the exception of GR (significant higher activity in organisms pre-exposed to the mixture at 100 μg/L compared to the control, $p < 0.05$) and GST (significant lower activity in exposure to the mixture at 10 μg/L with respect to the control, $p < 0.05$). No changes were observed in AChE activity and MT levels at the two exposure doses and both in the exposure and depuration phase (Fig. 2).

Biochemical responses have been treated jointly to obtain the numerical index IBR, reflecting an overview of the changes produced by exposure to pharmaceutical mixtures. In Table 1, IBR values at different exposure times and conditions are reported. IBR values increased during the first week of exposure (days 1 and 7) in organisms exposed to the pharmaceutical mixture compared to the control and this increase was greater at higher exposure dose. At the end of exposure phase (day 21) similar IBR values were obtained for both control and exposed organisms. After depuration phase (day 28) the IBR values obtained for control and organisms exposed to the highest exposure concentration of drug mixture were similar (and lower compared to IBR values obtained for the day 21). On the other hand, the IBR value for organisms exposed to the mixture at 10 μg/L was higher with respect to the control and similar to the value obtained at the end of exposure phase.

4.3. Gene expression

Temporal evolution of selected genes is shown in the Fig. 3. *Mnsod*, *gpx*, *gst*, *Thy* and *Colα* mRNA expression was significantly ($p < 0.05$) up-regulated after 7 days in hepatopancreas of crayfishes exposed to the mixture at 10 μg/L. Significant changes in gene expression after the first exposure week at the lowest mixture concentration is also confirmed by

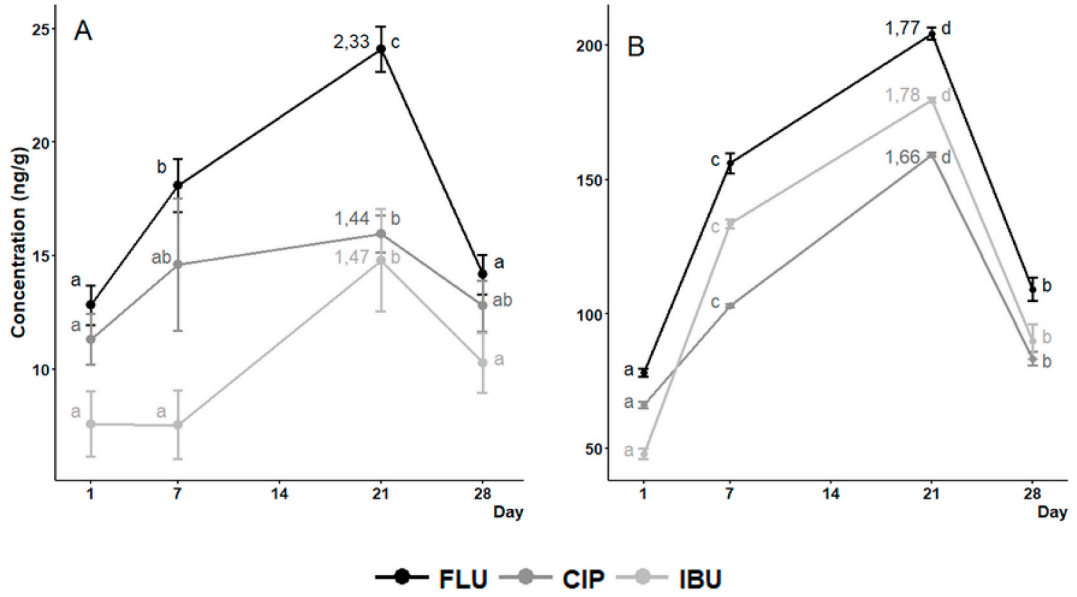

Fig. 1. Flumequine (FLU), ciprofloxacin (CIP) and ibuprofen (IBU) concentrations (ng/g wet weight) (mean ± standard deviation, n=3) in the hepatopancreas of *Procambarus clarkii* exposed to pharmaceutical mixture at 10 (A) and 100 (B) μg/L. For each compound, letters indicate statistically significant differences ($p < 0.05$) between different exposure times. Numbers represent the bioconcentration factor (L/Kg) corresponding to each compound at the end of the exposure phase (day 21, BCF_{21}).

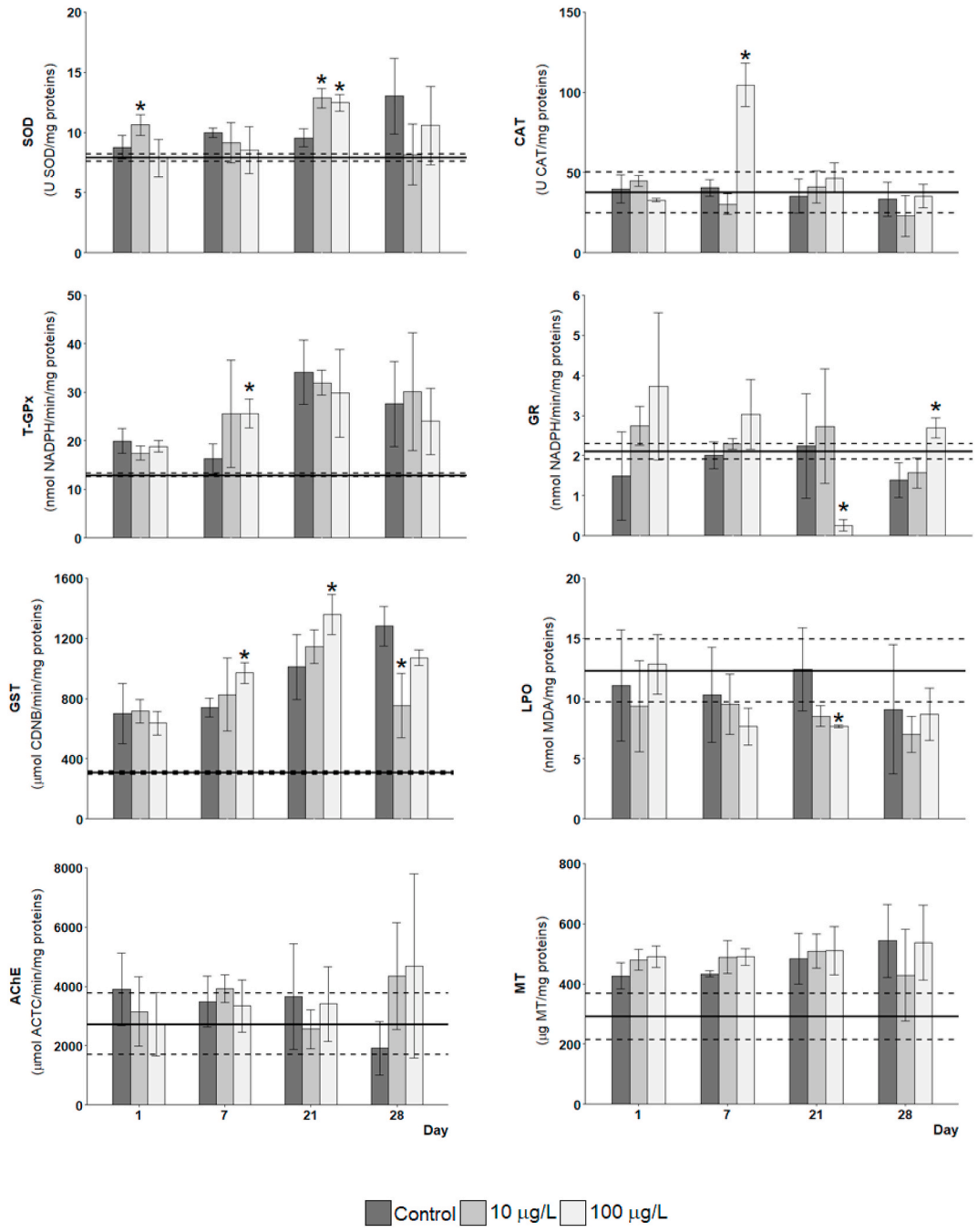

Fig. 2. Biochemical biomarkers in the hepatopancreas of *Procambarus clarkii* exposed to the pharmaceutical mixture at 10 and 100 µg/L. Values represent the mean ± standard deviation for n=3 replicates of pooled samples. Asterisks indicate significant differences ($p < 0.05$) from the control. Solid and dashed lines represent the value (mean ± standard deviation) for each biomarker at the day 0 (from the depuration tank).

Table 1
Integrated Biomarker Responses (IBR) values for biochemical responses in hepatopancreas of *Procambarus clarkii* at different experimental conditions (control and mixture at 10 and 100 µg/L) and exposure times (1, 7, 21 and 28 days). Asterisks indicate IBR values for control at the day 0 (organisms from the depuration tank).

Experimental condition	Exposure time				
	0d	1d	7d	21d	28d
Control	1.68*	5.49	5.63	11.47	7.69
10 µg/L		9.94	10.42	12.24	13.77
100 µg/L		16.26	17.23	15.18	6.62

the principal component analysis (Fig. 4B) that shows strong correlation between *Mnsod*, *gpx*, *gst*, *Thy*, *Colα*, *cat* and *SnAp* (0.74, 0.70, 0.83, 0.86, 0.78, 0.86 and 0.78 respectively) and the first dimension F1 (that explains 57.24% of the total variance), and positive association of these genes in crayfish exposed to 10 µg/Lat day 7.Expression levels for these genes were recovered at the end of exposure phase (day 21, no differences between control and exposed were observed). For the other genes no significant differences were observed with respect to the control throughout the exposure period. Similarly, at the end of depuration phase the expression levels of all genes under study did not show significant differences between control and organisms pre-exposed to the pharmaceutical mixtures.

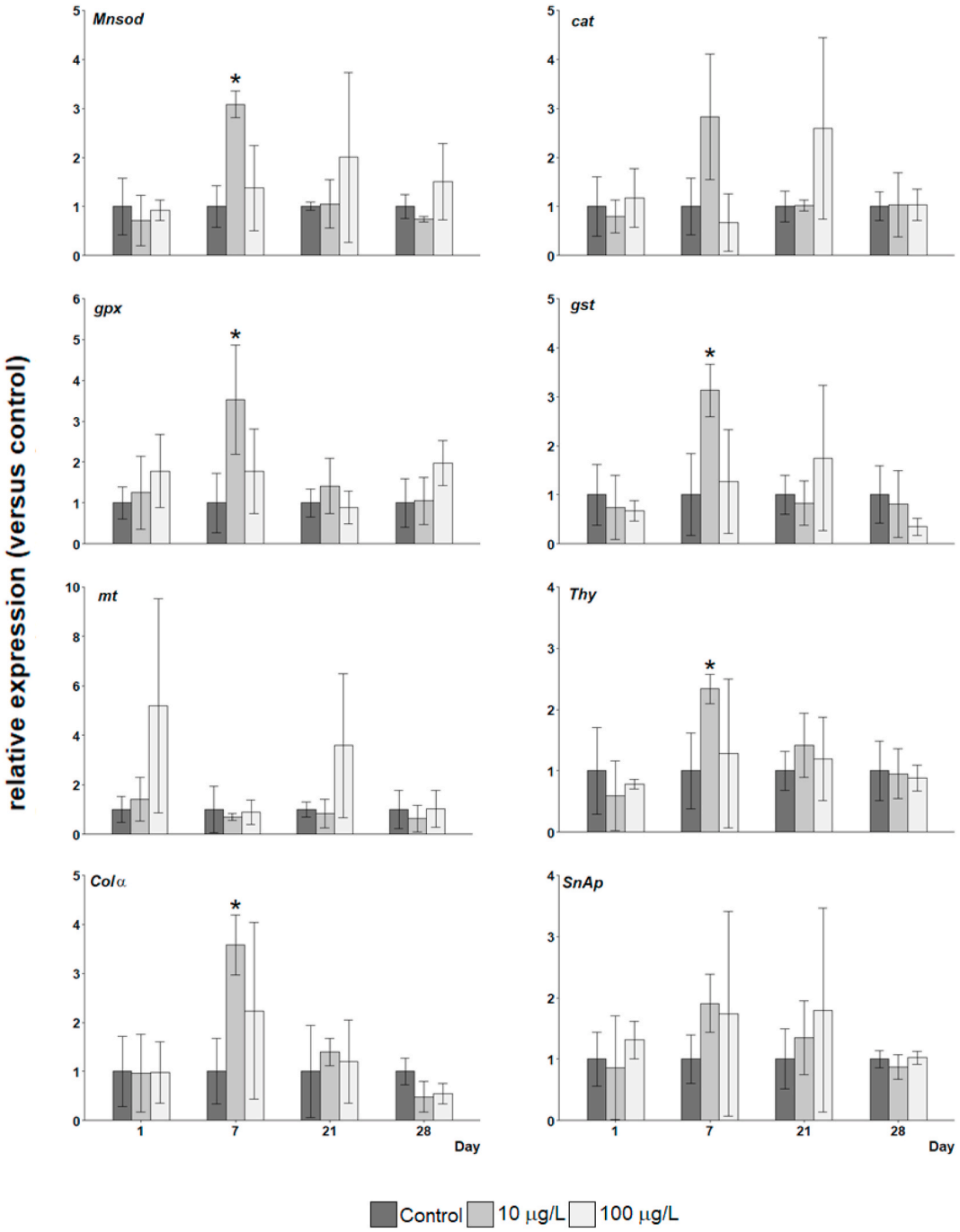

Fig. 3. Relative gene expression in hepatopancreas of *Procambarus clarkii* exposed to pharmaceutical mixture at 10 and 100 µg/L. Values represent mean ± standard deviation for n=3 replicates of pooled samples. Asterisks indicate significant differences ($p < 0.05$) from the control.

4.4. Proteomic

For each experimental condition, three biological replicates (each composed by proteins coming from 5 animals) were loaded. A total of 44 bands evenly distributed across the M*w* range were resolved in the gels (two representative gels used to determine changes in the protein abundance profiles caused by exposure to the drug cocktail in the samples collected after different time and doses are shown in Supplementary Fig. S5). Twenty-nine bands showed statistically significant changes in abundance ($p < 0.05$). In order to better understand the meaning of these differences, an analysis of principal components was applied to the data considering doses, times and intensity of the bands. The results are shown in Fig. 4C. The PCA analysis separated the control and the exposed animals and differentiated the groups corresponding to animals treated with each of the tested doses (0,10 µg/L and 100 µg/L). The first two components explained >60% of the total variation. PC1 (39%) separated control from drug cocktail exposed crayfish, and PC2 (26.3%) separated both exposure groups, pointing to the dose of 10 µg/L as the most effective.

Bands of interest were split and analyzed by MALDI-TOF-TOF for identification. Twenty-five protein bands were identified, corresponding to 22 different protein species (two bands were identified as GST and three others as ACT) (Table 2). Seven of the protein sequences obtained correspond to proteins of unknown function. Thirteen differentially expressed proteins increased their abundance with treatment as a function of dose and time of exposure. These include haemocyanin (HC,

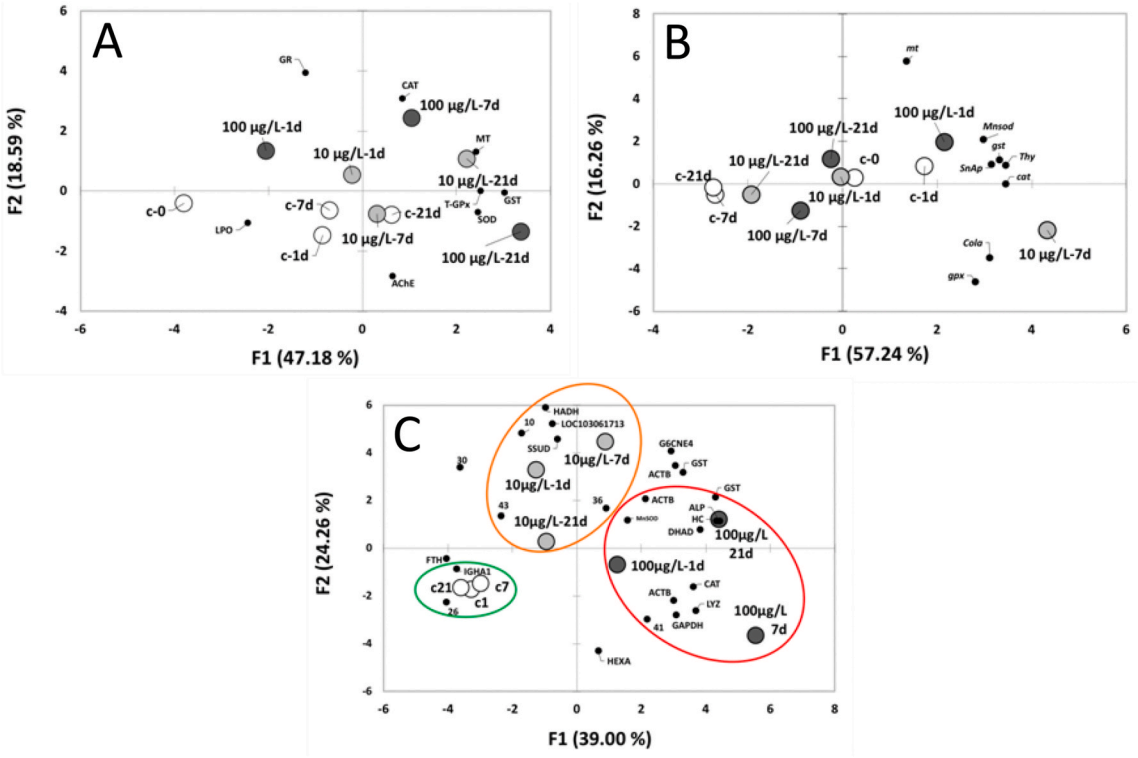

Fig. 4. PCA graphs based on biomarker responses (A), gene expression level (B) and protein abundance (C) in hepatopancreas of *Procambarus clarkii* during the exposure phase. The control (c) and pharmaceutical mixture concentrations (10 and 100 µg/L) are indicated for each sampling day (0, 1, 7 and 21 d).

bands 5 and 6); catalase (CAT, band 9); actin (ACT, bands 15, 23 and 24); glyceraldehyde 3-P dehydrogenase (GAPDH, band 22); superoxide dismutase (SOD, band 25); glutathione S-transferase (GST, bands 29 and 38); dihydroxy acid dehydrate (DHAD, band 32) and alkaline phosphatase (ALP, band 34). In contrast, immunoglobulin alpha-1 (IGHA1, band 19) and ferritin (FER, band 37) decreased their amount depending on the intensity of the treatment. The remaining proteins increased their levels at the dose of 10 µg/L but showed an irregular behavior at the higher tested dose. These results demonstrate that the drugs are being incorporated by *P. clarkii* and that even the environmentally relevant dose of 10 µg/L alters the proteostasis in the crayfish hepatopancreas and hence disturbs the animal's metabolism, as will be discussed later.

5. Discussion

The crayfish *P. clarkii* is considered as a good model organism for the evaluation of alterations produced by pollutants through *in vivo* laboratory assays (Antón et al., 2000). Ecotoxicological studies carried out to date focus mainly on the effects produced on this organism by metals and pesticides (Gago-Tinoco et al., 2014; Suárez-Serrano et al., 2010; Vioque-Fernández et al., 2009b; Zhao et al., 2019 among others) whereas the impact of pharmaceutical exposure is poorly investigated and most of works focus on psychoactive drugs (antiepileptics, anxiolytics, anaesthetics, etc.) and their effects on neurotransmission, movement and behaviour in *P. clarkii* (Cooper et al., 2001; Hossain et al., 2021; Majeed et al., 2015; Sewell et al., 1984 among others).

In this study, the selected pharmaceuticals accumulated progressively in the hepatopancreas throughout the exposure period. Uptake and bioconcentration of pharmaceuticals by aquatic organisms are mostly dependant on physico-chemical characteristics of exposure medium and properties of selected contaminant molecules. The octanol/water partition coefficient ($logK_{ow}$) can be used to predict the accumulation potential of pollutants by calculating a theoretical bioconcentration factor (BCF). According to the BCF values found in the existing literature, IBU, CIP and FLU ($logK_{ow}$-based BCF=3 L/kg for all compounds) are considered to have a low tendency to bioconcentrate in aquatic organisms (Franke et al., 1994; http://toxnet.nlm.nih.gov). BCF values obtained in this work for the hepatopancreas of *P. clarkii* at the end of exposure phase varied between 1.44 and 2.33 L/kg proving the very low accumulation potential for the three pharmaceuticals. Additionally, a theoretical accumulation order can be derived by $logK_{ow}$ values (IBU > FLU > CIP, according to the criteria that accumulation and $logK_{ow}$ are linearly related), or pKa (FLU > CIP > IBU, non-ionized molecules accumulate more easily than ionic forms and, at pH values typical of surface waters, molecular ionization increases with decreasing pKa) (Supplementary Table S1) (Barron, 1990; Daughton and Brooks, 2011). Results obtained in the assay with *P. clarkii* did not agree with theoretical predictions (with exception of FLU whose higher accumulation could be related to the greater abundance of non-ionized form in the aquatic medium). Besides physico-chemical properties of the exposure medium and selected chemicals, the biological characteristics of exposed organisms and contaminant dose and composition affect absorption and accumulation of pharmaceuticals (Arnot and Gobas, 2006; Barron, 1990; Ding et al., 2017; Smith et al., 2010).

Pharmaceutical accumulation in the hepatopancreas of *P. clarkii* showed to change depending on compound and exposure dose. BCF values obtained at day 21 indicate a higher accumulation of FLU in hepatopancreas of organisms exposed to mixture at 10 µg/L. Similar results were observed in molluscs (*Dreissena polymorpha* exposed to CBZ and IBU) and fish (*Onchorhynchus mykiss* exposed to IBU) that after exposure to increasing concentrations of pharmaceuticals exhibited higher BCF values at lower concentrations (Brown et al., 2007; Contardo-Jara et al., 2011). Additionally, a variation of accumulation pattern over time was observed at both exposure doses: the accumulation rate at 100 µg/L was continuous throughout exposure time whereas at 10 µg/L a different behavior for the three compounds was observed. Low or no-accumulation rate (as observed at 10 µg/L for CIP from day 1–21 or for IBU from day 1–7) may be due to metabolic and detoxification processes that counterbalance uptake of pharmaceuticals so that accumulation does not occur (Almeida et al., 2017; Garcia et al., 2012).

Table 2
Identification of proteins differentially expressed in the digestive gland of *Procambarus clarkii* exposed to a mixture of ibuprofen, flumequine and ciprofloxacin during different times. Asterisks indicate statistically significant changes in protein expression compared to the control.

Band number	Protein Name	Accession Number	Theor. Mw (kDa)	Exp. Mw (kDa)	Theor. pI	MOWSE score	Matching peptides	Sequence coberture (%)	Fragmented ions	Ion score	Protein symbol	Fold change (dose *vs* control)					
												10 μg/L			100 μg/L		
												Day 1	Day 7	Day 21	Day 1	Day 7	Day 21
1	**Hexokinase**	tr|F6JFS6|F6JFS	28.11	78.36	5.03	58	5	100	–	–	**HEXA**	−1.48*	1.08	3.46*	−1.16	1.80*	−1.04
5	**Hemocyanin B chain**	sp|P83180|HCYB_	65.53	66.00	5.81	190	12	27	LLEQHHWFSLFNTR DSYGYHLDR YGGEFPARPDNVHFEDVDGVAR	52 41 30	**HC**	1.18	1.49*	1.40	1.09	1.01	1.07
6	**Hemocyanin B chain**	sp|P83180|HCYB_	65.53	64.66	5.81	126	10	24	LLEQHHWFSLFNTR	76	**HC**	1.76*	2.57*	2.54*	3.20*	2.99*	3.04*
9	**Catalase**	tr|B2XQY0|B2XQY	59.30	54.79	6.63	148	7	17	GAGAFGYFEVTHDITK FNPFDLTK	70 50	**CAT**	1.03	1.25	1.00	1.00	2.07*	1.41
10	**Conserved hypothetical protein (membrane protein?)**	gi|224532894	254.47	52.01	4.74	101	31	16	–	–	**CHP**	1.44	1.18	1.00	−6.72*	6.56*	24.92*
11	**3-hydroxyacyl-CoA dehydrogenase**	gi|239995542	77.34	50.66	7.54	103	19	28	–	–	**HADH**	1.00	1.09	−1.30	−2.59*	3.33*	7.74*
12	**Alkanesulfonate monooxygenase**	gi|254562587	42.68	49.87	5.9	80	12	35	–	–	**SSUD**	1.00	1.34	−1.38	−1.13	1.29	−1.59*
14	**Putative viral A-type inclusion protein**	tr|G6CNE4|G6CNE	306.75	47.23	4.94	88	30	15	–	–	G6CNE4	1.00	−1.04	−2.09*	2.81*	2.94*	3.62*
15	**Actin**	gi|156773/AAA28321.1	42.15	44.29	5.22	175	8	29	AVFPSIVGRPR VAPEEHPVLLTEAPLNPK QEYDESGPSIVHR	10 83 35	**ACTB**	1.15	1.68*	1.00	1.19	1.75*	−1.26
17	**Chain A. Mutant Human Lysozyme**	gi|13399627/1I22_A	15.24	40.85	9.13	89	6	40	STDYGIFQINSR	48	**LYZ**	−1.71*	−3.27*	−1.04	7.92*	12.19*	22.80*
19	**Immunoglobulin heavy constant alpha 1**	gi|34527679/BAC85432.1	54.65	39.14	6.49	91	5	18	WLQGSQELPR QEPSQGTTTFAVTSILR	33 37	**IGHA1**	−1.25	1.30	1.05	−1.40	−1.91*	−37.65*
21	**Zinc finger protein 41-like protein**	gi|24559751/AAN61169.1	90.58	36.64	9.26	89	17	28	–	–	**LOC103061713**	−1.14	1.00	1.13	−8.85*	−12.62*	−28.85*
22	**Glyceraldehyde-3-phosphate dehydrogenase**	tr|Q7YT60|Q7YT6	35.97	35.20	6.54	242	9	41	IGINGFGR VPTPDVSVVDLTVR VVSWYDNEFGYSTR	18 97 67	**GAPDH**	−1.11	1.11	−1.01	4.87*	6.94*	−1.86*
23	**Actin beta/gamma 1**	tr|Q17C86|Q17C8/AAEL004616-PA	42.06	33.76	5.3	195	8	29	VAPEEHPVLLTEAPLNPK GYTFTTTAER SYELPDGQVITIGNER	97 30 21	**ACTB**	1.10	1.33	1.10	2.92*	3.43*	2.31*
24	**Gamma actin (partial)**	gi|21541270/CAD10659.1	17.31	31.00	5.06	126	5	44	VAPEEHPVLLTEAPINPK GYTFTTTAER	60 28	**ACTB**	1.10	1.05	1.10	1.88*	2.56*	2.66*
25	**Cytosolic manganese superoxide dismutase**	gi|194346532/ABX44762.3	31.51	30.46	5.44	95	8	29	AFFNVINWSNVNER	42	**MnSOD**	1.19	1.47*	3.02*	1.13	1.49*	2.44*
26	**Leucyl-tRNA synthetase**	gi|37679095	97.20	29.49	4.95	105	19	23	–	–	**LARS**	1.26	−1.43	−1.81*	1.47*	2.28*	128.46*
29	**Glutathione S transferase D1**	tr|F5A6F0|F5A6F0	25.43	23.55	4.97	92	9	37	SYAEYVYR	31	**GST**	1.25	1.33	1.26	1.20	1.55*	1.91*
30	**PREDICTED: nuclear mitotic apparatus protein 1**	gi|119907521	238.56	23.37	5.49	79	25	15	–	–	**NMAP1**	1.09	1.00	1.00	1.02	1.29	1.11
32	**Dihydroxy-acid dehydratase**	gi|186473830	66.94	21.94	5.73	84	14	27	ITPGMLMAAMR	3	**DHAD**	11.52*	−1.32	1.09	1.97*	−1.04	−15.17*
34	**Alkaline phosphatase**	gi|161727372/BAF94313.1	12.30	21.28	10.01	82	9	70	–	–	**ALP**	2.13*	2.75*	4.07*	15.30*	17.23*	23.45*

(*continued on next page*)

Table 2 (*continued*)

Band number	Protein Name	Accession Number	Theor. Mw (kDa)	Exp. Mw (kDa)	Theor. pI	MOWSE score	Matching peptides	Sequence coberture (%)	Fragmented ions	Ion score	Protein symbol	Fold change (dose *vs* control)					
												10 µg/L			100 µg/L		
												Day 1	Day 7	Day 21	Day 1	Day 7	Day 21
36	**GA27520. isoform A**	tr|B5DWH7|B5DWH	48.36	20.69	9.58	77	13	36	–	–	**GA27520**	−2.08*	−1.25	1.32	−1.43	−1.56*	−1.55*
37	**Ferritin**	tr|F5A6F3|F5A6F	19.44	20.21	5.15	384	9	50	QNYHEDCEAAINK NTDAHLTNMLEEDFLEEQVESIEK AGTSGLGEFLFDK AGTSGLGEFLFDKELK	68 55 55 130	**FER**	2.10*	1.89*	−5.03*	−6.67*	−5.26*	0.00
38	**Glutathione S transferase D1**	tr|F5A6F0|F5A6F	25.43	18.03	4.97	71	10	33	–	–	**GST**	3.85*	1.07	−1.04	−1.32	−2.44*	−2.31*
41	**DNA ligase**	gi|228904740	73.81	16.34	5.28	85	13	19	KEIEAFIK	26	**LIG**	1.17	1.31	−1.53*	3.66*	4.55*	2.88*
43	**Uncharacterized protein involved in ubiquinone biosynthesis**	gi|85708522	29.72	15.19	6.81	87	11	53	–	–	**UBS**	1.10	1.06	1.23	1.00	−2.01*	−2.02*

Furthermore, we can suppose that high exposure levels cause an imbalance between the accumulation and elimination processes, producing a progressive accumulation of drugs along the time.

The decrease in the level of pharmaceuticals accumulated in hepatopancreas after 7 days of depuration indicates the ability of *P. clarkii* to carry out regulatory processes (metabolization and excretion) that allow eliminating these compounds and the existence of these processes is confirmed by the presence of IBU, CIP and FLU in the water at the end of the depuration phase (Almeida et al., 2017).

It is well known that the presence of low levels of pollutants in the aquatic medium as well as their low bioaccumulation is not indicative of the absence of ecotoxicological risk for organisms. Most pharmaceuticals have shown to induce adverse effects in aquatic organisms at very low concentrations (ng-µg/L) (Almeida et al., 2020; Fabbri and Franzellitti, 2015; Parolini, 2020, among others).

Proteomic methods evaluate modifications in protein abundance profiles that can be used as biomarkers of contamination while providing data on the toxicity mechanisms of contaminants. The selected approach (1-DE SDS-PAGE) allowed us to have a global vision of the hepatopancreas proteome of *P. clarkii* exposed to different doses of the mixture. The hepatopancreas is a multifunctional and vital organ for crustaceans with a key role in metabolic processes, nutrient uptake and immune functions (Roszer, 2014). Additionally it is considered a target organ for many environmental stressors and is involved in accumulation and detoxification processes (Zhang et al., 2019).The proteomic analysis identified 22 different proteins whose abundance changed in the hepatopancreas after the pharmaceutical cocktail exposure, depending on time and/or dose. The functional analysis of the differentially expressed proteins showed alterations in main cell functions: biotransformation and detoxification of xenobiotics, cytoskeleton homeostasis, carbohydrate metabolism and immune response. As discussed below, the alteration of these specific groups of proteins is probably linked to the ability of IBU, FLU and CIP to generate reactive oxygen species (ROS) in the cell and the subsequent onset of an antioxidant response (Bartoskova et al., 2013; Gonzalez-Rey and Bebianno, 2011; Kashida et al., 2006; Nunes et al., 2018; Zivna et al., 2015). Some of the proteins identified in this untargeted proteomic approach form part of the classical biomarkers battery that we choose to perform in this work, confirming the adequacy of our selection.

In the category of biotransformation and detoxification of xenobiotics 5 proteins (23%) are included: CAT (band 9), SOD (band 25) and GST (bands 29) increased their concentration, while SSUD (band 12) and UBS (band 43) diminished in abundance in treated crayfish. These alterations were particularly relevant at the highest exposure dose.

Glutathione S-transferases (GSTs) are essential Phase-II detoxification enzymes, which catalyze the conjugation of a variety of endogenous/exogenous compounds with reduced glutathione (GSH) for their excretion (Hayes and Strange, 2000). Two bands, 29 and 38 (Table 2) were identified as Glutathione S-transferase D1 in our study. However, the M*w* of band 38 suggests that it corresponds to a fragment of the original protein and then, changes in its concentration probably do not reflect variations in its associated function. Both the amount of GST protein and its activity (Table 2, band 29 and Fig. 2) increased with the concentration of the drug cocktail and the exposure time. Increased activity of GST has been described in *Carcinus maenas, Ruditapes philippinarum* and zebrafish *Danio rerio* exposed to IBU (Aguirre-Martinez et al. 2013, 2016, 2016; Bartoskova et al., 2013). GST activation has been also associated with FLU and CIP exposures (Kashida et al., 2006; Nunes et al., 2018), evidencing that the metabolism of the selected drugs requires the activation of the glutathione conjugation pathway.

Cells are protected against ROS generated by pollutants through a battery of enzymes such as CAT, SOD or GPxs (Bartoskova et al., 2013; Gonzalez-Rey and Bebianno, 2011; Kashida et al., 2006; Nunes et al., 2018; Zivna et al., 2015). The combined proteomic approach and the study of enzyme activities showed increased amounts and activity of CAT, SOD and T-GPx (only activity for the latter) in the hepatopancreas

of treated *P. clarkii* (Table 2 and Fig. 2), as expected from their respective roles in the cells: to detoxify peroxides and anion superoxide. For GST, CAT, SOD and GPx, alterations were also observed at transcriptional level: all genes encoding for these enzymes were over-expressed after 7 d of exposure at the mixture at 10 μg/L (Fig. 3). An enhanced transcription of CAT and SOD was also observed in the digestive gland of *D. polymorpha* exposed to low IBU concentrations (20 and 200 μg IBU/L after 1 day for CAT, 0.2 and 2 μg IBU/L after 4 days for SOD) (Contardo-Jara et al., 2011). Moreover, IBU exposure induced up-regulation of GST genes in *Daphnia magna* (5 and 50 μg/L) and *Crassostrea gigas* (1 and 100 μg/L); in oyster, similarly to our study, the most important changes were observed at the lowest exposure concentration and the authors suggested the induction of a hormesis-like effect with a low-dose transcriptional stimulation and high-dose inhibition to explain the gene expression trend (Serrano et al., 2015; Wang et al., 2016). Milan and coauthors (2013) analyzed the transcriptomic responses to IBU (100 and 1000 μg/L) in *R. philippinarum* and observed up-regulation of a number of genes involved in xenobiotic metabolism, including different forms of GST. Disruption of pathways associated with glutathione and xenobiotics metabolism as a consequence of down regulation of various GSTs genes was observed by Pomati et al. (2007) in zebrafish liver cells exposed to a pharmaceutical cocktail including IBU and CIP at environmentally relevant concentrations. It should be noted that changes in protein abundance, mRNA transcript levels and enzyme activity in GST, CAT and SOD are not parallel under all circumstances. Antioxidant defense responses can be regulated at different levels, transcriptional, translational, and post-translational, and these alterations can occur at the same time or independently of each other, depending on the pollutant, dose and time of exposure, generating different patterns of gene transcription levels, protein abundance and enzyme activities (Defo et al., 2015; Regoli et al., 2011; Zheng et al., 2016a, b). Increased levels of gene expression without post-transcriptional changes under low contamination conditions can represents a pre-adaptation mechanism through which, if the contaminant concentration increases or extends over time, organisms would be prepared to increase the activity of antioxidant defenses when they need it (Pillet et al., 2019). This mechanism could be related to the cellular content of ROS; we can hypothesize that, under low contamination conditions, the intracellular ROS level (produced in normal metabolic pathways such as processes involved in the control of pollutant accumulation and excretion) may increases reaching levels which are not high enough to produce oxidative stress (and consequently activation of antioxidant defense system) but sufficient to induce transcriptional factors that regulate the expression of different genes.

The detoxification and antioxidant mechanisms activated in the crayfish hepatopancreas appear to be effective in controlling the ROS internal content and prevent oxidative damage, as indicated by low LPO levels (similar or even lower with respect to the control, Fig. 2) throughout the entire exposure phase at both exposure doses. IBU induces alterations in enzymatic activity and possible oxidative damage in the digestive gland/hepatopancreas of different freshwater (*Corbicula fluminea*) and marine (*R. philippinarum* and *C. maenas*) organisms exposed to 50 μg IBU/L during a period similar to this study (Aguirre-Martínez et al. 2013, 2015, 2016, 2018). CIP showed to induce alterations in the antioxidant system of microalgae *Pseudokirchneriella subcapitata* and *Chlamyidomonas mexicana* (induction of CAT, SOD and GPx activities), and in the aquatic plant *Lemna minor* (CAT induction) when present at concentrations in the order of mg/L (Gomes et al., 2017; Nie et al., 2013; Xiong et al., 2017). Additionally, Gust et al. (2012) recorded increased ROS levels in the haemocytes of the bivalve *Elliptio complanata* exposed to 2500 ng CIP/L. In the existing literature no data were found about the induction of oxidative stress by FLU in aquatic organisms. However, this compound is able of generating ROS through its metabolic pathways and consequently induces oxidative stress in hepatic mice cells (Kenmochi, 2007).

Proteins are a target of oxidation and ROS can modify them and modulate both gene expression and proteins abundance (Deavall et al., 2012; Lushchak, 2011). In the pathogenesis of liver injury (hepatic fibrosis, often related to oxidative stress) in mammalian, a critical step is the activation of stellate cells associated to increased levels of collagen (type I and III) (Lee et al. 1995, 2001, 2001; Li et al., 2016; Shi and Rockey, 2017). According to these information, we can speculate that the increase in collagen expression observed in this study could be related to an initial inflammatory process in the hepatopancreas tissues induced by pharmaceutical exposure. We found here increased levels of HEXA and GAPDH, two enzymes involved in carbohydrate metabolism. HEXA is the enzyme responsible for the phosphorylation of glucose to glucose 6-phosphate and the initiation of glucose metabolism through both glycolysis and the pentose phosphate pathway (PPP). GAPDH oxidizes the triose glyceraldehyde 3-phosphate (G-3P), the product of glycolysis, fructose catabolism, PPP and glycerol metabolism so that G-3P follows its path to become pyruvate. Increased glucose metabolism has been detected in crustaceans such as *C. maenas* or *Homarus americanus* after pollutant exposure and has been associated to ROS clearance (Lorenzon et al., 2004; Wang et al., 2017). The increased HEXA activity observed in treated crayfish might activate the oxidative pentose phosphate pathway to generate NADPH and contribute to maintaining the GSH levels (Tang et al., 2015), affected by excess ROS generated by drug cocktail exposure.

Activation of glucose metabolism in crustacean under oxidative stress conditions is regulated by the crustacean hyperglycemic hormone, CHH, and abundant neuropeptide that stimulated lipid mobilization from the storage tissues (e.g., the hepatopancreas) including free fatty acids release into haemolymph, to be used metabolically (Lorenzon et al., 2004; Santos et al., 1997). The levels of 3-hydroxyacyl-CoA dehydrogenase (HADH) resulted increased in the hepatopancreas of *P. clarkii* after the drug cocktail exposure. This enzyme is essential for catalyzing the penultimate reaction of β-oxidation. HADH upregulation might reflect alterations of the lipids signaling pathways in the control of insulin secretion (Prentki, 1996).

The most intense band detected in the gels in all experimental conditions (Supplementary Fig. S5) was identified as hemocyanins (HC, bands 5 and 6), and their levels resulted increased in the crayfish exposed to the pharmaceutical mixture. HCs are copper-containing respiratory proteins, synthesized in the hepatopancreas, with essential roles in innate immunity. As an invertebrate, *P. clarkii* has no adaptive immunity and HCs provide an immediate and rapid immune response to invading microorganisms or other stressing situations, including exposure to pollutants (Hoffmann and Reichhart, 2002; Qin et al., 2018). Jebali and coauthors (2014) observed an induction of this protein in the crab *C. maenas* after transplantation in an area contaminated by hydrocarbons and metals and related it with regulation of immune system and oxygen transport activity. The levels of lysozyme (LYZ), one of the most important non-specific immune indicators, also resulted increased by the treatment. These results suggest that HCs and LYZ are inducible and up-regulated in response to this combination of drugs, confirming that both proteins participate in the innate immune response in *P. clarkii* (Fernández-Cisnal et al., 2014). Ferritin (FER) has a key role in iron homeostasis and also participates in many biological processes, including oxidation and immune response (Theil, 1987). Up-regulated expression of the ferritin mRNA has been reported in the hepatopancreas of crustaceans after viral infection and in crabs from polluted areas (urban, industrial and agricultar contamination) (Ghedira et al., 2016; Nayak et al., 2011; Zhang et al., 2006). However, in our study FER resulted increased only by low doses and short times (Table 2). In any other case, a substantial diminution in protein abundance was detected. In agreement with our results, several studies have reported the down-regulation of ferritin mRNA in crustaceans after exposure to oxidant elements like metals or pesticides (Osuna-Jiménez et al., 2014; Poynton et al., 2011; Taylor et al., 2013). In previous work Fernández-Cisnal et al. (2017) reported decreased FER protein levels in the hepatopancreas of pollution exposed *P. clarkii* individuals paralleled by

clear signs of oxidative stress and significant deregulation of proteins involved in the immune response. By using redox proteomics, the same authors proposed that the decrease in FER levels was caused by a substantial increase in the oxidation status of this protein that results in aggregates rapidly removed as part of the antioxidant defense strategy (Fernández-Cisnal et al., 2014; Welch et al., 2002).

Overall, the results obtained in proteomic, transcriptional and biochemical analyses suggest that the cocktail of IBU, CIP and FLU generates oxidative stress in the hepatopancreas of *P. clarkii*. An antioxidative stress response is consequently elicited that derives glucose to oxidative PPP, seeking to obtain the NADPH needed to maintain the levels of GSH used by several biotransforming (i.e., GST) and antioxidant enzymes (i.e., SOD, CAT). An uneven effect was apparently exerted over different components of the crayfish immune response, since HC and LYZ increased, but other proteins such as IGHA1 and FER diminished with the dose and the time. However, at least in the case of FER, diminution might be consequence of its sensitivity to oxidation and further degradation. Down-regulation of FER by the pharmaceutical cocktail might potentiate their toxicity, given that FER is also considered as part of the antioxidant response.

6. Conclusions

Our study provides data about the toxicity of a mixture of ciprofloxacin, flumequine and ibuprofen on the crayfish *Procambarus clarkii* contributing to improve the knowledge regarding the interaction between pharmaceuticals and aquatic organisms. Pharmaceuticals are accumulated in the hepatopancreas and, despite the low accumulation potential shown, induced alterations at different levels of biological organization. Changes in molecules related with antioxidant and detoxification processes, both from the point of view of gene expression, protein abundance and enzymatic activity indicate that an alteration of the redox status and the activation of mechanisms to reduce the accumulated drugs occurs in the hepatopancreas of the crayfish.

The assessment of classical biomarkers as well as the expression of genes, both selected a priori, is useful to detect specific alterations (i.e. oxidative stress or neurotoxicity) in organisms exposed to environmental contaminants. On the other hand, proteomics, as large-scale study of proteins, provides information about the cell's protein composition of a selected biological system and its variation under different exposure conditions, allowing us to unravel mechanisms of action of selected contaminants and biological processes and/or pathways affected by them.

Results obtained in this study support the usefulness of evaluating the selected responses over time (medium or long term) to observe the evolution of health status of the organism (evolution of effects at different biological organization levels, recovery, induction of permanent damage, etc.). In the case of *P. clarkii* we observed that it was capable of overcoming the alterations related to the oxidative stress and, after removal of pharmaceuticals from the exposure medium (depuration), have a redox state similar to control organisms.

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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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111396.

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