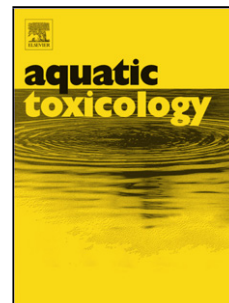


Accepted Manuscript

Title: Effects of single and combined exposure of pharmaceutical drugs (carbamazepine and cetirizine) and a metal (cadmium) on the biochemical responses of *R. philippinarum*



Authors: Ângela Almeida, Vânia Calisto, Valdemar I. Esteves, Rudolf J. Schneider, Amadeu M.V.M. Soares, Etelvina Figueira, Rosa Freitas

PII: S0166-445X(18)30135-8
DOI: <https://doi.org/10.1016/j.aquatox.2018.02.011>
Reference: AQTOX 4859

To appear in: *Aquatic Toxicology*

Received date: 29-11-2017
Revised date: 10-2-2018
Accepted date: 14-2-2018

Please cite this article as: Almeida, Ângela, Calisto, Vânia, Esteves, Valdemar I., Schneider, Rudolf J., Soares, Amadeu M.V.M., Figueira, Etelvina, Freitas, Rosa, Effects of single and combined exposure of pharmaceutical drugs (carbamazepine and cetirizine) and a metal (cadmium) on the biochemical responses of *R. philippinarum*. *Aquatic Toxicology* <https://doi.org/10.1016/j.aquatox.2018.02.011>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Effects of single and combined exposure of pharmaceutical drugs
(carbamazepine and cetirizine) and a metal (cadmium) on the biochemical responses
of *R. philippinarum*

Ângela Almeida¹, Vânia Calisto², Valdemar I. Esteves², Rudolf J. Schneider³,
Amadeu M. V. M. Soares¹, Etelvina Figueira¹, Rosa Freitas¹

¹Biology Department & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

²Chemistry Department & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

³BAM Federal Institute for Materials Research and Testing, Richard-Willstaetter -Str.
11, D-12489 Berlin, Germany

***Corresponding author:** Rosa Freitas

Address: Departamento de Biologia, Universidade de Aveiro Campus
Universitário de Santiago 3810-193 Aveiro, Portugal e-mail address: rosafreitas@ua.pt

Highlights

- Combined treatments led to differences in drug uptake and effects in clams
- The uptake of CTZ in clams was favored by the presence of Cd
- Combined treatments showed smaller effects (in LPO and activities of GSTs) than exposures to a single contaminant

ABSTRACT

In the aquatic environment, organisms are exposed to complex mixtures of contaminants which may alter the toxicity profile of each compound, compared to its toxicity alone. Pharmaceutical drugs (e.g. carbamazepine (CBZ) and cetirizine (CTZ)), and metals (e.g. cadmium (Cd)), are among those contaminants that co-occur in the environment. However, most studies concerning their toxicity towards aquatic species are based on single exposure experiments. Thus, the present study aimed to evaluate single and combined effects of Cd and CBZ or CTZ (single conditions: Cd, CTZ, CBZ; combined conditions: CTZ+Cd, CBZ+Cd) on biomarkers related to oxidative stress and energy metabolism in the edible clam *Ruditapes philippinarum*, by exposing the organisms for 28 days to environmentally relevant concentrations of these contaminants. The biomarkers studied were: i) the electron transport system activity, protein and glycogen content (indicators of organisms' metabolic status); ii) lipid peroxidation and the ratio between reduced and oxidized glutathione (indicators of oxidative stress); iii) superoxide dismutase and catalase activities (indicators of

antioxidant defence) and iv) activities of glutathione S-transferases (indicator of biotransformation capacity). Results obtained showed that the uptake of Cd and CBZ was not affected by the combined presence of the contaminants. However, for CTZ, the uptake was higher in the presence than in the absence of Cd. Concerning toxicity data in general, the combined exposures (CTZ+Cd, CBZ+Cd) had lower biological effects than the contaminants alone. Nevertheless, our data showed that despite the low concentrations tested, they were enough to exert biological effects that differed between single and combined treatments, evidencing the need to conduct more co-exposure studies to increase the environmental relevance of the gathered data.

Keywords: Biomarkers, pharmaceutical drugs, metals, mixtures, invertebrates

1. INTRODUCTION

Aquatic systems are commonly exposed to a variety of contaminants, and in most cases, with well-known toxic impacts to inhabiting organisms (La Farre et al., 2008; Wu et al., 2016). In fact, in the aquatic environment, organisms are subjected to complex mixtures of contaminants which may interact and exert effects different from single compound exposures, including cumulative, antagonistic or synergistic effects, depending on the type of contaminant and its mode of action (Bound and Voulvoulis, 2004). Pharmaceutical drugs and metals are examples of contaminants that frequently co-occur in aquatic systems (Andreu et al., 2016; Damásio et al., 2011; Martins et al., 2014; Tchounwou et al., 2012). However, current toxicological studies on these contaminants are mostly focused on single exposures, and toxicity studies evaluating the interactive effects between drugs and metals are scarce, with only few studies available (Alsop and Wood, 2013; Cleuvers, 2003; Pires et al., 2016a, 2016b).

Among pharmaceutical drugs, carbamazepine (CBZ, an antiepileptic) and cetirizine (CTZ, an antihistamine) have been detected in the aquatic environment. CBZ was reported in aquatic bodies at concentrations ranging between 0.03 and 11.6 µg/L (Bahlmann et al., 2012, 2009; Calisto et al., 2011; Loos et al., 2009; Metcalfe et al., 2003; Sacher et al., 2001; Ternes, 1998), while CTZ has been detected in water bodies, mainly at concentrations in the ng/L range (Bahlmann et al., 2012, 2009; Bebianno et al., 2016; Calisto et al., 2011; Kosonen and Kronberg, 2009; Larsson et al., 2007; Nödler et al., 2014, 2011). Nevertheless, higher CTZ concentrations were found by Bahlmann et al. (2012) in surface waters of the Teltowkanal channel (Berlin, Germany) (up to 0.72 µg/L) and by Bebianno et al. (2016) in an effluent from a psychiatric hospital in Montpon (France) (up to 1.28 µg/L). Recent studies have demonstrated the toxic effects of both drugs to marine species, including bivalves. Among them, oxidative stress has been reported as an impact of CBZ following its accumulation and/or metabolization in this group of organisms (e.g. Aguirre-Martínez

et al., 2016; Almeida et al., 2017a, 2015, 2014; Contardo-Jara et al., 2011; Freitas et al., 2016, 2015b; Juhel et al., 2017; Martin-Diaz et al., 2009; Tsiaka et al., 2013). Regarding CTZ, fewer studies have investigated the toxic potential of this drug (Almeida et al., 2017b; Bergheim et al., 2014; Borowska et al., 2016; Letullier et al., 2014; Li, 2013; Rittschof et al., 2003; Teixeira et al., 2017) but oxidative stress response was also identified following its uptake by bivalves (Teixeira et al., 2017). Nevertheless, up to now the majority of studies concerning CBZ and CTZ toxicity to aquatic organisms (including bivalves) have focused on the individual effects of each compound. Regarding bivalves, a limited number of studies evaluated the combined effect of CBZ with other classes of drugs or other contaminants (Di Poi et al., 2017; Juhel et al., 2017; Mohamed et al., 2017) and no studies exist concerning the combined presence of CTZ. For example, Juhel et al. (2017) studied the effects of CBZ, the plasticizer bisphenol A and the herbicide atrazine in the green mussel *Perna viridis* after 7 days of exposure to individual and mixture conditions. Biomarkers related with neurotoxicity, immunotoxicity, genotoxicity and detoxification enzymes were evaluated. The authors observed that the mixture of the three contaminants acted generally in an additive manner on the biomarkers tested at environmentally relevant concentrations.

As previously referred, in the aquatic environment pharmaceutical drugs do not occur isolated but in combination with other types of contaminants, including metals. Cadmium (Cd), among other metals (mercury, lead, nickel), is in the list of priority substances in the field of water policy (Annex I of the Directive 2013/39/EU). This metal has been detected in low concentrations in marine waters, ranging from 0.005 to 0.110 µg/L (EPA 2016). However, higher concentrations were detected in estuaries and costal zones (La Colla et al., 2015; Vicente-Martorell et al., 2009). For example, Vicente-Martorell et al. (2009) detected total Cd concentrations ranging from 0.7 to 8.9 µg/L in the water of the Ría de Huelva estuarine system in Spain. Other studies,

due to the proximity of agriculture and industrial areas, found higher Cd concentrations in water bodies (Fatima et al., 2015; Moradi et al., 2017; Morin et al., 2008). The toxicity that Cd poses to aquatic organisms is a well-known issue, including oxidative stress impacts in bivalves (e.g. D'costa et al., 2017; Dovzhenko et al., 2005; Figueira et al., 2012b; Geret et al., 2002; Jo et al., 2008). Although there is a high diversity of studies evaluating the impacts of single exposures to Cd, a limited number of works assessed the impacts induced by the mixture of Cd with other metals (e.g., Meyer et al., 2015; Rouchon and Phillips, 2017; Traudt et al., 2016), some of them being performed in bivalves (Bigot et al., 2011; Marie et al., 2006; Spann et al., 2011; Xie et al., 2016). Considerably fewer studies have investigated the impacts induced by the combination of this metal with pharmaceutical drugs (Li et al., 2011; Ragusa et al., 2017), and the effects on bivalves are still not explored.

Despite the available toxicity data for Cd, CBZ and CTZ, to the best of our knowledge, no studies evaluated the combined effects of these contaminants in bivalves. Thus, the present study aimed to evaluate the single (Cd, CTZ, CBZ) and combined (CTZ+Cd, CBZ+Cd) effects of these contaminants on the oxidative stress status and energy metabolism in the edible clam *Ruditapes philippinarum*, by exposing the organisms for 28 days to environmentally relevant concentrations.

2. MATERIALS AND METHODS

2.1 Experimental conditions

In the present study, the clam *Ruditapes philippinarum* (Adams & Reeve, 1850) was used as model organism. Bivalves, such as *R. philippinarum* have been applied in toxicological studies due to their filter feeding capacity and sessile life style which makes them excellent model organisms to infer the effects of toxic compounds present in the aquatic environment (Rittschof and McClellan-Green, 2005). Clams were collected in the Ria de Aveiro, an estuarine system located in the Northwest Atlantic coast of Portugal, in the Mira channel, a southern arm of this ecosystem considered non-contaminated (Cerqueira and Pio, 1999). For laboratory experiments, clams with similar size (mean length: 4.2 ± 0.3 cm; mean width: 3.0 ± 0.2 cm) were selected.

Clams were maintained in the laboratory for 15 days before testing, for depuration and acclimation to laboratory conditions (Freitas et al., 2012; Maffei et al., 2009). During this period, organisms were maintained at $17.0 \pm 1.0^\circ\text{C}$, pH 7.80 ± 0.10 , 12 light:12 dark photoperiod and continuous aeration in artificial seawater (salinity 25 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center). Seawater was renewed every two days and the clams were not fed.

After acclimation, clams were submitted to a chronic exposure (28 days) to Cadmium (Cd), Cetirizine (CTZ) and Carbamazepine (CBZ) according to the following conditions: Control (CTL, 0 $\mu\text{g/L}$ Cd, 0 $\mu\text{g/L}$ CTZ, 0 $\mu\text{g/L}$ CBZ); Cd (0.5 $\mu\text{g/L}$); CTZ (0.6 $\mu\text{g/L}$); CBZ (1.0 $\mu\text{g/L}$); CTZ+Cd (0.6 $\mu\text{g/L}$ CTZ + 0.5 $\mu\text{g/L}$ Cd); CBZ+Cd (1.0 $\mu\text{g/L}$ CBZ + 0.5 $\mu\text{g/L}$ Cd).

The concentrations of CBZ and CTZ used were selected taking into consideration measurements at the Ria de Aveiro as well as in other aquatic systems worldwide (see references in the Introduction section). In the Ria de Aveiro, Calisto et al. (2011) quantified CBZ and CTZ in surface water at concentrations of 0.1 and 0.04 $\mu\text{g/L}$, respectively. Also, contamination with CBZ and CTZ was recorded in wastewaters from

two main WWTPs belonging to the Aveiro region at concentrations around 0.6 µg/L and 0.3 µg/L, respectively. However, higher concentrations were found in other surface waters. For example, Bahlmann et al. (2009) determined CBZ concentrations in surface water of the Teltowkanal channel (Berlin), ranging between 0.75 and 3.2 µg/L. Also, Bahlmann et al. (2012) found CTZ concentrations up to 0.72 µg/L in the same channel (Teltowkanal). Cd has also been detected in Ria de Aveiro. Monterroso et al. (2007) found Cd at concentrations of 0.12 µg/L in the water column of Laranjo Bay (at the Ria de Aveiro). Cd was also identified in Ria de Aveiro: in sediment samples (Velez et al. (2015a) 0.20 µg/g dry weight, in particulate matter (Monterroso et al. (2003) 1.5 µg/g, and accumulated in inhabiting organisms such as bivalves (Velez et al. (2015a) 0.15 µg/g fresh weight and fish (Cid et al. (2001) < 0.043 µg/g fresh weight).

In the present study, for each condition 72 clams were used, with 3 containers per condition and 4 individuals per container filled with 3L of artificial seawater (salinity 25 ± 1 g/L). During the exposure period (28 days) containers were submitted to continuous aeration, temperature $18 \pm 1^\circ\text{C}$ and 12:12 h (light/dark) photoperiod. Animals were fed with Algamac protein plus (150 000 cells/animal) twice a week. The exposure medium was renewed once a week (7 days exposure period) and the concentrations re-established. Mortality was checked daily, and organisms were considered dead when their shells gaped and failed to shut again after an external stimulus.

Seawater standards of CBZ and CTZ at the same concentrations as used in the assay and exposed to the same conditions but without organisms (blanks) were also prepared to evaluate possible drug loss during the experiments through adsorption onto the test vessels and/or photodegradation. Cadmium and mixture (Cd+CBZ and Cd+CTZ) blanks were also prepared, with the same concentration as used in the assay and exposed in the same conditions but without organisms.

At the end of the exposure, organisms were individually frozen and mechanically pulverized in a mill with liquid nitrogen. Each homogenized organism was divided in 0.3

g aliquots fresh weight (FW), further used for biomarker analyses and contaminant quantification (Cd, CTZ and CBZ).

2.2 Contaminant quantification

2.2.1 Carbamazepine and Cetirizine concentrations

The tissue samples (0.3 g FW aliquots) of 6 organisms per condition were used to determine CBZ and CTZ concentrations, for which extractions were performed in deionized water (1:2, w/v). Samples were sonicated for 15 s at 4 °C, centrifuged for 20 min at 10 000 g (4 °C) and the supernatants collected for analyses.

To evaluate the behavior of the drugs in the exposure medium and in blanks during the assay, water aliquots were collected each week of exposure immediately after contamination (beginning) and immediately before the water renewal (end) for quantification. Water aliquots from the controls were also collected at the same sampling periods and analyzed.

CBZ and CTZ were quantified by a direct competitive ELISA (Enzyme-Linked Immunosorbent Assay), according the procedure developed by Bahlmann et al. (2012) and Calisto et al. (2011) with the modifications described in Almeida et al. (2014). The same ELISA procedure was used to quantify both pharmaceuticals. Due to the cross-reactivity of CTZ against the monoclonal antibody used for detecting CBZ, CTZ can be quantified with the procedure used for detecting CBZ (Calisto et al., 2011). A sample buffer at pH 7.6 was selected for quantifications considering the similar affinity of the antibody towards CBZ and CTZ at this pH. Considering that the exposure experiments did not include samples with both pharmaceuticals, the verified cross-reactivity was not a drawback for this work, allowing both drugs to be quantified individually. The absorbance was measured on a microplate spectrophotometer at 450 nm and referenced to 650 nm. All samples and standards (0 - 100 µg/L) were determined in triplicate on each plate. A four-parametric logistic equation (Findlay and Dillard, 2007)

was fitted to the mean values of the standards in order to obtain a calibration curve. For the analysis of the clam tissues, the standards were prepared in ultrapure water by diluting a 10 mg/L stock solution of CBZ or CTZ (also prepared in ultrapure water). For the analysis of water samples, the standards were prepared in artificial seawater (25 g/L NaCl) by diluting a stock solution of CBZ or CTZ with the same concentration.

2.2.2 Cadmium concentrations

For Cd quantification in clams, 0.3 g FW aliquots of previously homogenized tissue (3 organisms per condition), were freeze-dried and then digested with 4 mL of nitric acid (HNO_3 65%) at 60°C for 18 h. After that, 2 mL of hydrogen peroxide (H_2O_2 37%) were added and left to stand for 1 h at 80°C. At the end of this time the mixture was evaporated almost to dryness, 0.4 mL of 65% HNO_3 was added and left to stand for 15 min. The obtained digested solutions were collected in 10 mL of ultrapure water.

To evaluate the behavior of Cd in the exposure medium and in blanks during the assay, aliquots of water were collected immediately after contamination (beginning) and immediately before (end) the water renewal at each week of exposure. Water samples (exposure medium, controls and blanks) for Cd quantification were acidified with 0.1 mL of 65% HNO_3 .

The quantification of Cd in clam tissues and water samples (exposure medium and blanks) was performed by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry), in a certified laboratory (LCA) at the University of Aveiro. The calibration curve was obtained using IV-ICPMS 71A standard. The quality control was assured by running procedural blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-2 (Lobster hepatopancreas reference material for trace metals) in parallel with samples. Blanks were below the quantification limit and mean percentage of recovery for Cd was between 90 and 110%.

2.3 Biochemical markers

Biomarkers were determined in whole soft tissue of 6 organisms per condition (2 organisms per vessel, 3 vessels per condition, 6 organisms per condition). For each biochemical determination, 0.3 g FW per organism was used. For each condition, indicators of metabolic capacity (electron transport system activity, ETS), energy reserves (total protein content, PROT; glycogen content, GLY), oxidative stress (lipid peroxidation levels, LPO; ratio of reduced (GSH) to oxidized (GSSG) glutathione, superoxide dismutase activity, SOD; catalase activity, CAT), and biotransformation (enzyme activity of glutathione S-transferases, GSTs) were determined. The extraction for each biomarker was performed in specific buffers in the proportion 1:2 w/v. For ETS activity determination, samples were extracted in 0.1 M Tris-HCl pH 8.5, 15% (w/v) polyvinylpyrrolidone (PVP), 153 μ M magnesium sulphate (MgSO_4) and 0.2% (v/v) Triton X-100. For GLY, PROT, SOD, CAT and GSTs determinations, the extraction was done with potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM ethylenediaminetetraacetic acid (EDTA); 1% (v/v) Triton X-100; 1% (w/v) PVP; 1 mM dithiothreitol (DTT); pH 7.0). For LPO determination, samples were extracted using 20% (w/v) trichloroacetic acid (TCA). For GSH and GSSG determination samples using 0.6% sulfosalicylic acid in potassium phosphate buffer (0.1 M dipotassium phosphate; 0.1 M potassium dihydrogen phosphate; 5 mM EDTA; 0.1% (v/v) Triton X-100; pH 7.5). Samples were homogenized for 15 s at 4 °C and centrifuged for 20 min at 10 000 g (or 3000 g for ETS) (4 °C). Supernatants were stored at -80 °C or immediately used. All biochemical parameters were determined in duplicate. All measurements were done using a microplate reader (Biotek).

2.3.1 Indicators of metabolic capacity and energy reserves

ETS activity was determined based on King and Packard (1975) methodology with the modifications presented by De Coen and Janssen (1997). The samples were incubated with buffered substrate solution (0.13 M Tris-HCl, 0.3% (v/v) Triton X-100, pH 8.5), NAD(P)H (1.7 mM NADH and 250 μ M NADPH) and 8 mM *p*-IodoNitroTetrazolium. The mixture was shaken and the absorbance was measured at 490 nm during 10 min in 25 s intervals. The amount of formazan formed was calculated using $\epsilon=15\,900\text{ M}^{-1}\text{cm}^{-1}$ and the results expressed in nmol/min/g FW.

GLY content was quantified based on the sulphuric acid method DuBois et al. (1956), using glucose standards (0 - 10 mg/mL). Samples and standards were incubated with 5% (v/v) phenol and 98% sulfuric acid, shaken and incubated at room temperature for 30 min. Following this step, absorbance was measured at 492 nm. The results were expressed in mg/g FW.

The Biuret method of Robinson and Hogden (1940) was applied to determine PROT content. Bovine serum albumin (BSA) was used as standard (0 - 40 mg/mL). Biuret reagent was added to the samples. The mixture was shaken and left to incubate at 30 °C for 10 min, after which absorbance was measured at 540 nm. The results were expressed in mg/g FW.

2.3.2 Indicators of oxidative stress

LPO was measured by the quantification of TBARS (ThioBarbituric Acid Reactive Substances) according to Ohkawa et al. (1979). Samples were incubated with thiobarbituric acid (TBA) and TCA for 25 min at 96 °C. After that, absorbance was measured at 535 nm ($\epsilon=156\text{ mM}^{-1}\text{cm}^{-1}$). LPO levels were expressed in nmol of MDA equivalents formed per g FW.

The quantification of GSH and GSSG was performed based on Rahman et al. (2006) methodology, using GSH and GSSG standards (0 - 90 μ mol/L). For GSH determination KPE and DTNB (Ellman's reagent) was added to the samples and

standards. The mixture was shaken and the absorbance was measured at 412 nm. For GSSG determination, samples and standards were first derivatized with 2-vinylpyridine for 1 h. After this, triethanolamine was added and left to stand for 10 min. Then, the mixture was incubated in KPE, glutathione reductase, DTNB (1:1, v/v) and NADPH. The mixture was shaken for few seconds and the absorbance was measured at 412 nm. The ratio GSH/GSSG was obtained by dividing GSH content by 2*GSSG content.

Antioxidant and biotransformation enzyme activities

SOD activity was determined based on the method described by Beauchamp and Fridovich (1971), using SOD standards (0 - 60 U/mL). Samples and standards were incubated with 56.1 mU/mL xanthine oxidase and the reaction buffer (pH 8.0;

0 mM Tris-HCl, 0.1 mM diethylene triamine pentaacetic acid (DTPA), 0.1 mM hypoxanthine and 68.4 μ M nitro blue tetrazolium (NBT)). After that, absorbance was measured at 560 nm. The enzymatic activity was expressed in U/g FW (U= 1 μ mol/min).

CAT activity was quantified according to Johansson and Borg (1988). Formaldehyde standards (0-150 μ M) were used for the standard curve. The samples and standards were incubated for 20 min with 50 mM potassium phosphate buffer (pH 7.0), methanol and 35.28 mM hydrogen peroxide. To stop this reaction, 10 M potassium hydroxide was added, followed by 34.2 mM Purpald and the mixture incubated for 10 min. After this, 65.2 mM potassium periodate was added, and the mixture left to incubate for 5 min. Following this step, absorbance was measured at 540 nm. The enzymatic activity was expressed in U/g FW (U =1 nmol/min).

The activity of GSTs was quantified following Habig et al. (1974). The samples were incubated in a reaction solution consisting of 60 mM 1-Chloro-2,4-dinitrobenzene (CDNB) and 10 mM GSH in 0.1 M potassium phosphate buffer at pH 6.5 (0.1 M dipotassium phosphate, 0.1 M potassium dihydrogen phosphate). The mixture was

shaken for a few seconds and the absorbance was measured at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) for 10 min in 25 s intervals. The enzymatic activity was expressed in U/g FW (U = 1 $\mu\text{mol/min}$).

2.4 Data and statistical analyses

The bioconcentration factor (BCF) (Arnot and Gobas, 2006) for each condition was determined by dividing the concentration of CBZ and CTZ present in clam tissues by an average of the concentrations in the exposure medium obtained for the entire exposure period. For Cd, however, the concentration in the clam tissues was divided by the nominal concentration, since it was not possible to detect Cd levels in the exposure medium with good precision (Cd concentrations are lower than the quantification limit).

Cd, CBZ and CTZ concentrations in soft tissues of clams and in the exposure medium/blanks, as well as BCF and biochemical parameters obtained from each tested condition were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008). A one-way hierarchical design was followed in this analysis. The pseudo-F p-values were evaluated in terms of significance. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: a) for drug and metal concentration in the exposure medium and blanks, no significant differences exist between the average concentrations in the water aliquots at the time immediately after spiking (beginning) and the average concentrations in the water aliquots just before weekly water renewals (end) for each condition (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd); b) for drug and metal concentrations in clam tissues, BCF and biomarker responses, no significant differences existed between CTL and Cd, CTL and CBZ (only for biomarkers), CTL and CTZ (only for biomarkers), CTL and CTZ+Cd (only for biomarkers), Cd and CTZ+Cd, CTZ+Cd and CTZ, CTL and CBZ (only for biomarkers),

CTL and CBZ+Cd (only for biomarkers), CBZ+Cd and Cd, and CBZ+Cd and CBZ. For the drug quantifications (exposure medium, blanks, clam tissues), BCF and biomarkers the significance values are presented in Tables 2, 4 and 5. Significant differences ($p \leq 0.05$) when existing are highlighted in bold. Also, in Figures (1, 2, 3 and 4) and Tables (1 and 3), significant differences ($p \leq 0.05$), when existing, were represented with different letters.

3. RESULTS

3.1 Mortality

After 28 days of exposure none of the tested conditions induced mortality.

3.2 Drug and metal concentrations in water samples

Drug concentrations in the exposure media and blanks showed that, for each drug treatment (CTZ, CTZ+Cd, CBZ, CBZ+Cd), no significant changes occurred comparing the average concentrations measured at the beginning of the exposure weeks and the mean concentrations obtained at the end of the exposure weeks of exposure (Tables 1 and 2). Moreover, for the same collection period and drug no differences were observed in drug concentration comparing single and combined treatments (Tables 1 and 2). Since drug concentrations in the exposure medium and blanks were generally constant over the exposure period and the spiked concentrations were similar to the nominal concentrations, these results validated the spiking methodology and the assay duration (Tables 1 and 2).

The concentration of Cd in the exposure medium and blanks in the different treatments (Cd, CTZ+Cd, CBZ+Cd) was lower than the quantification limit ($< \text{LOQ}$ of 2 $\mu\text{g/L}$) (Table 1).

3.3 Drug and metal concentrations in clam tissues and BCF

The results of metal concentration in clam tissues (Tables 3 and 4) showed that in Cd treatments (Cd, CTZ+Cd, CBZ+Cd) the metal concentration significantly increased in clam tissues compared to the control condition, but no significant differences occurred concerning Cd concentrations and BCF values between single and combined treatments (Cd vs CTZ+Cd; Cd vs CBZ+Cd) (Tables 3 and 4).

The results on drug concentration and BCF in clam tissues (Tables 3 and 4) showed that the CTZ concentration and the corresponding BCF was significantly higher

in clams exposed to the combined treatment (CTZ+Cd) in comparison with the single treatment (CTZ). On the other hand, no significant differences in tissue concentrations and BCF were observed comparing single (CBZ) and combined (CBZ vs CBZ+Cd) treatments for CBZ exposed clams.

3.4. Biochemical markers

3.4.1 Indicators of metabolic capacity and energy reserves

Regardless of the treatment, clams presented no significant differences in the ETS activity after 28 days of exposure (Figure 1A and Table 5). Nevertheless, slightly higher mean ETS values were observed in clams exposed to CBZ and CBZ+Cd in comparison to control values and clams exposed to Cd (Figure 1A).

Regarding GLY content, although higher mean values were observed in clams exposed to Cd, no significant differences were observed among treatments (Figure 1B and Table 5).

Similarly, PROT content appeared higher in clams exposed to Cd in comparison to control but the differences among treatments were not statistically significant (Figure 1C and Table 5).

3.4.2 Indicators of oxidative stress

After 28 days of exposure significantly higher LPO levels were observed in clams exposed to CBZ in comparison to control values (Figure 2A and Table 5). No significant differences in LPO levels were observed among the remaining treatments although in clams exposed to Cd the mean of LPO was higher than in control clams (Figure 2A and Table 5).

GSH/GSSG values showed no significant differences among tested treatments, with the highest mean values observed in clams exposed to CBZ (Figure 2B and Table 5).

The activity of SOD was not significantly different among treatments, with the highest mean values in clams exposed to CBZ (Figure 3A and Table 5).

No significant differences in CAT activity was observed among treatments, with the highest mean values in clams exposed to CBZ (Figure 3B and Table 5).

The activity of GSTs was significantly lower in clams exposed to CTZ+Cd and CBZ in comparison to control clams (Figure 4 and Table 5). The activity of GSTs was significantly higher in clams exposed to CTZ in comparison to those exposed to CTZ+Cd, while GST activity tended to be higher in clams exposed to the combination of CBZ+Cd in comparison to single CBZ exposure (Figure 4 and Table 5).

4. DISCUSSION

In the present study, the uptake of drugs (CBZ and CTZ) and Cd, as well as the effects induced on *R. philippinarum* were evaluated after chronic exposures to the individual contaminants (Cd, CBZ, CTZ) and their mixtures (CBZ+Cd, CTZ+Cd) to understand if the combined effect of each drug with Cd would induce a different impact in clams than each contaminant acting alone.

The results of the metal uptake showed no significant changes in clams exposed to Cd alone and combined with both pharmaceuticals, indicating that the presence of the drugs did not influence the uptake of the metal. Previous studies with *R. philippinarum* demonstrated the capacity of clams to accumulate this metal under laboratory conditions (Blasco and Puppo, 1999; Figueira et al., 2012b; Wang et al., 2011; Zhao et al., 2014), although in these cases the concentrations tested were not environmentally relevant. For example, Blasco and Puppo (1999) showed an uptake of Cd of ~50 and ~150 µg/g DW after exposure of *R. philippinarum* clams to 200 and 600 µg/L for 7 days, respectively, while Wang et al. (2011) observed increased uptake of Cd in gills (up to ~18 µg/g DW) and in digestive gland (up to ~49 µg/g DW) of *R. philippinarum* submitted to 15 µg/L of the metal with the increase of exposure period (21 days). When Cd was combined with other contaminants, previous studies showed that the pattern of metal accumulation in bivalves could be altered. Wang et al. (2011) studied the combined effects of Cd and benzo[α]pyrene (BaP) (Cd: 15 µg/L, BaP: 0.01 µg/L) in the gills and digestive gland of *R. philippinarum* exposed for 21 days, observing that the accumulation of Cd was higher in the combined treatments compared to the single exposure. On the contrary, Marie et al. (2006) showed a decrease in Cd uptake after the exposure of the clam *Corbicula fluminea* and the mussel *Dreissena polymorpha* to Cd and Zinc (exposure concentrations: 0.133 µM (= 14.9 µg/L) Cd + 15.3 µM (= 1 mg/L) Zinc) for 24 days. The authors observed that, under the combined exposure of Cd and Zinc, the metals could interact in absorption,

sequestration and excretion processes. The present findings may indicate that neither CBZ nor CTZ influenced Cd uptake, which may be related to fact that neither drug influenced the filtration rate of clams. Furthermore, the present study seems to indicate that CBZ and CTZ may not establish interactions with Cd interfering with the entrance of the metal to the clam. Nevertheless, recently Andreu et al. (2016) showed significant direct correlations between the presence of Ni and diazepam, norfloxacin, ofloxacin and fenofibrate, and inverse relationships with ibuprofen under environmental conditions. Therefore, different impacts on metal accumulation may not only depend on the metal and its exposure concentrations, but also on the mixture of contaminants (and their concentrations) and species used.

Concerning the effects exerted by Cd (individually or combined with the drugs used) on clam biomarkers, a significant decrease of GST activity was observed in clams exposed to Cd and especially to Cd+CTZ, suggesting an inhibitory effect of the metal on the activity of this enzyme. Only a few studies have evaluated the activity of GSTs in bivalves exposed to Cd under laboratory experiments. However, this was done in other invertebrate species (Cunha et al., 2007; Won et al., 2011). For example, Cunha et al. (2007), among other biomarkers studied, observed an increase in GST activity at the highest concentration of Cd (1.4 mg/L) but an inhibition at lower concentrations (LOEC = 0.044 mg/L) in the marine gastropod *Nucella lapillus*. These authors hypothesized that GST inhibition could have resulted from a direct action of Cd on the enzyme; an indirect effect of ROS induced by Cd on the enzyme or a depletion of GSH and downregulation of GST genes. Nevertheless, our results showed that, apart from changes observed in GST activity, the presence of Cd did not cause changes in the markers of metabolic status, cellular damage and antioxidant defense either when the metal was acting individually or as a mixture with pharmaceuticals. Previous studies showed, however, that Cd affects the energy reserves and metabolic activity and exerts oxidative stress in bivalves. Among other studies, research

conducted by Bebianno et al. (1993) reported that the increase in PROT content in *Ruditapes decussata* clams exposed to Cd (400 µg/L, 40 days exposure) could have been related with the induction of metallothionein (MT) synthesis. Metallothioneins are low molecular weight heat-stable cytoplasmic proteins, rich in cysteine residues, involved in Cd complexation in bivalve species (Bebianno et al., 1993; Bebianno and Serafim, 1998; Figueira et al., 2012a, 2012b, Velez et al., 2016, 2015b; Wang et al., 2011). Concerning oxidative stress markers, Dovzhenko et al. (2005) demonstrated that Cd induced LPO in *Modiolus modiolus* mussels through damage to the antioxidant system, decreasing its capacity to inactivate free radicals. Concerning the activity of antioxidant enzymes, D'costa et al. (2017) observed an increased CAT activity in the clam *Meretrix casta* exposed to environmental concentrations of Cd (0.75 – 3 µg/L) for 15 days. With regard to the interactions between toxic metals and pharmaceutical drugs only few studies have been done, with no works conducted in bivalves. Ragusa et al. (2017) evaluated the individual and combined effects of the antimicrobial agent, sulfamethoxazole, and Cd in *Paracentrotus lividus* sea urchin embryos, showing block of gastrulation, weakening of cell-cell junction and reduction of survival, when the metal-pharmaceutical combination was compared with the metal effects alone. Overall, our findings demonstrated that Cd uptake was similar in metal only and metal-pharmaceutical exposures, which resulted in similar biochemical responses in clams exposed to Cd alone or to a mixture of the metal with CBZ and CTZ. Since other studies identified different results comparing the effects induced by the co-exposure of Cd and pharmaceutical drugs (Li et al., 2011; Ragusa et al., 2017) and other organic compounds (Wang et al., 2011) with the exposure to the single compounds, it appeared that Cd effects on the aquatic organisms may depend on the type of contaminants present in the exposure medium, namely in aquatic systems.

Concerning pharmaceutical uptake, the results obtained in this work showed that: i) in CBZ-exposed clams the accumulation was similar both without and with the

metal (CBZ+Cd) indicating that no interaction occurred between the two contaminants (Cd and CBZ), ii) in CTZ-exposed clams the uptake was significantly higher in the combined treatment (CTZ+Cd) as compared to the pharmaceutical only exposure, indicating that the presence of Cd enhanced CTZ accumulation. Possibly different uptake mechanisms, metabolization processes and/or subcellular accumulation may be the reason for the obtained results with CBZ and CTZ in the presence of Cd.

Concerning toxicological responses to the drugs, significant changes were only observed for LPO content and GST activity. Our results showed that i) in comparison to control, clams exposed to CBZ showed significantly more cell damage (LPO) but the activity of biotransformation enzymes (GSTs) was significantly lower, while in clams exposed to CBZ+Cd no significant changes were observed; ii) clams exposed to CBZ+Cd showed significantly higher GST activity in comparison to CBZ-exposed clams. The enzymatic activities of CBZ+Cd-exposed animals were closer to those of control animals; iii) clams exposed to CTZ alone showed no significant changes in comparison with control, while in CTZ+Cd-exposed clams, a significant decrease in GST activity was observed; iv) clams exposed to CTZ+Cd also showed significantly lower GSTs activity in comparison to CTZ alone exposure. These data suggest that the combined treatments with drugs and Cd exerted fewer biological effects compared to single drug exposures, indicating that the presence of the metal attenuated the drug effects. Concerning oxidative stress markers, previous studies showed oxidative stress caused by CBZ in bivalves (Almeida et al., 2015, 2014, Freitas et al., 2016, 2015a, 2015b; Martin-Diaz et al., 2009) and to smaller extent by CTZ (Teixeira et al., 2017). Specifically, CBZ has been linked to the induction of LPO in bivalves (Aguirre-Martínez et al., 2015; Almeida et al., 2015, 2014; Freitas et al., 2015a). In bivalves exposed to CBZ, previous studies generally reported an induction of antioxidant enzymes to eliminate ROS generated due to drug accumulation and biotransformation. For example, Almeida et al. (2015) observed an increase in SOD activity in *R.*

philippinarum exposed to 0.03 – 9.0 µg/L of CBZ for 28 days. Concerning the activity of biotransformation enzymes, previous studies generally show an increase or maintenance of GST activity in bivalves exposed to CBZ, which is not in accordance with the results of the present work (e.g., Aguirre-Martínez et al., 2015; Almeida et al., 2015; Freitas et al., 2015a, 2015b; Juhel et al., 2017; Martin-Díaz et al., 2009). However, Almeida et al. (2014) observed a decrease of GST activity in *R. philippinarum* exposed to CBZ (0 – 9 µg/L) for 28 days, indicating that lower levels of GSH were available to participate in the conjugation reactions and consequently, GSTs were not involved in the phase II biotransformation of CBZ. Concerning CTZ, scarce information is available on the impacts on bivalves (Almeida et al., 2017b; Teixeira et al., 2017), although Teixeira et al. (2017) found the development of an oxidative stress response in the mussel *M. galloprovincialis* exposed to this drug (0.3 – 12 µg/L, 28 days exposure).

As previously reported, the combined effects of CBZ or CTZ with Cd in bivalves have not earlier be studied. However, some authors evaluated the effects of pharmaceutical drugs when combined with other drugs or other contaminants. For example, Gonzalez-Rey et al. (2014) observed an antagonistic effect of copper (Cu) combined with pharmaceutically active products (ibuprofen, diclofenac and fluoxetine) on the activity of SOD in the mussel *Mytilus galloprovincialis* exposed for 15 days. The authors saw an inhibition of SOD activity that resulted in the depletion of CAT substrate. On the other hand, Franzellitti et al. (2015) observed an increase in CAT activity in the mussel *M. galloprovincialis* exposed to the combination of fluoxetine and propranolol for 7 days, suggesting that the change of the antioxidant defense activity was based on the increased accumulation of fluoxetine induced by the presence of propranolol. Pires et al. (2016a), studied the combined effects of CBZ and caffeine in *H. diversicolor* polychaete for 28 days, and observed an antagonistic effect on LPO levels exerted by the combination of CBZ and caffeine. However, the combined effects

of the drugs were generally similar to the effects exerted by the drugs when acting alone.

ACCEPTED MANUSCRIPT

5. CONCLUSIONS

The present study showed that the combination of pharmaceutical drugs (CBZ and CTZ) and Cd exerted different responses from those of the compounds alone, either in the uptake (only for CTZ clams) or in the biomarker responses. Uptake of CTZ was affected by the presence of the metal. For CBZ, no changes in uptake occurred in the presence of Cd. Despite the low exposure concentrations tested, these were enough to exert some biological effects (significant changes on LPO content and GST activity), which were different when the compounds acted alone and when in combination. This is particularly important since in the environment the organisms are exposed to complex mixtures, providing a more realistic approach and complementing the scarce literature about this issue.

Acknowledgments

Vânia Calisto benefited from a post-doc grant (SFRH/BPD/78645/2011) and Ângela Almeida benefited from a PhD grant (SFRH/BD/110218/2015) given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia. Rosa Freitas benefited from a research position funded by the Integrated Programme of SR&TD “Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate” (reference Centro-01-0145-FEDER-000018), co-funded by Centro 2020 program, Portugal 2020, European Union, through the European Regional Development Fund. Thanks are also due, for the financial support to CESAM (UID/AMB/50017), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

References

- Aguirre-Martínez, G.V., DelValls, A.T., Martín-Díaz, M.L., 2015. Yes, caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen have an effect on *Corbicula fluminea* (Müller, 1774). *Ecotoxicol. Environ. Saf.* 120, 142–154.
- Aguirre-Martínez, G.V., DelValls, T.A., Martín-Díaz, M.L., 2016. General stress, detoxification pathways, neurotoxicity and genotoxicity evaluated in *Ruditapes philippinarum* exposed to human pharmaceuticals. *Ecotoxicol. Environ. Saf.* 124, 18–31.
- Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M., Figueira, E., Freitas, R., 2017a. Toxicity associated to uptake and depuration of carbamazepine in the clam *Scrobicularia plana* under a chronic exposure. *Sci. Total Environ.* 580, 1129–1145.
- Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M., Figueira, E., Freitas, R., 2017b. Ecotoxicity of the antihistaminic drug cetirizine to *Ruditapes philippinarum* clams. *Sci. Total Environ.* 601, 793–801.
- Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M., Figueira, E., Freitas, R., 2014. Presence of the pharmaceutical drug carbamazepine in coastal systems: effects on bivalves. *Aquat. Toxicol.* 156, 74–87.
- Almeida, Â., Freitas, R., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M., Figueira, E., 2015. Chronic toxicity of the antiepileptic carbamazepine on the clam *Ruditapes philippinarum*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 172, 26–35.
- Alsop, D., Wood, C.M., 2013. Metal and pharmaceutical mixtures: Is ion loss the mechanism underlying acute toxicity and widespread additive toxicity in zebrafish? *Aquat. Toxicol.* 140, 257–267.
- Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. Permanova + for PRIMER. Plymouth UK Primer-E.

- Andreu, V., Gimeno-García, E., Pascual, J.A., Vazquez-Roig, P., Picó, Y., 2016. Presence of pharmaceuticals and heavy metals in the waters of a Mediterranean coastal wetland: Potential interactions and the influence of the environment. *Sci. Total Environ.* 540, 278–286.
- Arnot, J.A., Gobas, F.A., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environ. Rev.* 14, 257–297.
- Bahlmann, A., Carvalho, J.J., Weller, M.G., Panne, U., Schneider, R.J., 2012. Immunoassays as high-throughput tools: Monitoring spatial and temporal variations of carbamazepine, caffeine and cetirizine in surface and wastewaters. *Chemosphere* 89, 1278–1286.
- Bahlmann, A., Weller, M.G., Panne, U., Schneider, R.J., 2009. Monitoring carbamazepine in surface and wastewaters by an immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem.* 395, 1809–1820.
- Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44, 276–287.
- Bebianno, M.J., Nott, J.A., Langston, W.J., 1993. Cadmium metabolism in the clam *Ruditapes decussata*: the role of metallothioneins. *Aquat. Toxicol.* 27, 315–333.
- Bebianno, M.J., Serafim, M.A., 1998. Comparison of metallothionein induction in response to cadmium in the gills of the bivalve molluscs *Mytilus galloprovincialis* and *Ruditapes decussatus*. *Sci. Total Environ.* 214, 123–131.
- Bebianno, M.J., Sroda, S., Gomes, T., Chan, P., Bonnafe, E., Budzinski, H., Geret, F., 2016. Proteomic changes in *Corbicula fluminea* exposed to wastewater from a psychiatric hospital. *Environ. Sci. Pollut. Res.* 23, 5046–5055.
- Bergheim, M., Gminski, R., Spangenberg, B., Dębiak, M., Bürkle, A., Mersch-Sundermann, V., Kümmerer, K., Gieré, R., 2014. Recalcitrant pharmaceuticals in the aquatic environment: a comparative screening study of their occurrence,

- formation of phototransformation products and their in vitro toxicity. Environ. Chem. 11, 431–444.
- Bigot, A., Minguez, L., Giambérini, L., Rodius, F., 2011. Early defense responses in the freshwater bivalve *Corbicula fluminea* exposed to copper and cadmium: transcriptional and histochemical studies. Environ. Toxicol. 26, 623–632.
- Blasco, J., Puppo, J., 1999. Effect of heavy metals (Cu, Cd and Pb) on aspartate and alanine aminotransferase in *Ruditapes philippinarum* (Mollusca: Bivalvia). Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 122, 253–263.
- Borowska, E., Bourgin, M., Hollender, J., Kienle, C., McArdell, C.S., Von Gunten, U., 2016. Oxidation of cetirizine, fexofenadine and hydrochlorothiazide during ozonation: Kinetics and formation of transformation products. Water Res. 94, 350–362.
- Bound, J.P., Voulvoulis, N., 2004. Pharmaceuticals in the aquatic environment—a comparison of risk assessment strategies. Chemosphere 56, 1143–1155.
- Calisto, V., Bahlmann, A., Schneider, R.J., Esteves, V.I., 2011. Application of an ELISA to the quantification of carbamazepine in ground, surface and wastewaters and validation with LC–MS/MS. Chemosphere 84, 1708–1715.
- Cerqueira, M.A., Pio, C.A., 1999. Production and release of dimethylsulphide from an estuary in Portugal. Atmos. Environ. 33, 3355–3366.
- Cid, B.P., Boia, C., Pombo, L., Rebelo, E., 2001. Determination of trace metals in fish species of the Ria de Aveiro (Portugal) by electrothermal atomic absorption spectrometry. Food Chem. 75, 93–100.
- Cleuvers, M., 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicol. Lett. 142, 185–194.
- Contardo-Jara, V., Lorenz, C., Pflugmacher, S., Nützmann, G., Kloas, W., Wiegand, C., 2011. Exposure to human pharmaceuticals Carbamazepine, Ibuprofen and

- Bezafibrate causes molecular effects in *Dreissena polymorpha*. *Aquat. Toxicol.* 105, 428–437.
- Cunha, I., Mangas-Ramirez, E., Guilhermino, L., 2007. Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*). *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 145, 648–657.
- Damásio, J., Barceló, D., Brix, R., Postigo, C., Gros, M., Petrovic, M., Sabater, S., Guasch, H., de Alda, M.L., Barata, C., 2011. Are pharmaceuticals more harmful than other pollutants to aquatic invertebrate species: a hypothesis tested using multi-biomarker and multi-species responses in field collected and transplanted organisms. *Chemosphere* 85, 1548–1554.
- D'costa, A., Shyama, S.K., PraveenKumar, M.K., Furtado, S., 2017. Genotoxic and Biochemical biomarker responses in *Meretrix casta* exposed to environmentally relevant concentrations of Cadmium. *Journal of Biosensors, Biomarkers and Diagnostics* 2(1), 1–7.
- De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular energy allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. *J. Aquat. Ecosyst. Stress Recovery* 6, 43–55.
- Di Poi, C., Costil, K., Bouchart, V., Halm-Lemeille, M., 2017. Toxicity assessment of five emerging pollutants, alone and in binary or ternary mixtures, towards three aquatic organisms. *Environ. Sci. Pollut. Res.* 1–13.
- Dovzhenko, N.V., Kurilenko, A.V., Bel'cheva, N.N., Chelomin, V.P., 2005. Cadmium-induced oxidative stress in the bivalve mollusk *Modiolus modiolus*. *Russ. J. Mar. Biol.* 31, 309–313.

- DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P. t, Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.
- EPA 2016. Aquatic life ambient water quality criteria- Cadmium- 2016. U.S. Environmental Protection Agency, Office of Water. Washington, D.C. EPA-820-R-16-002
- Fatima, M., Usmani, N., Firdaus, F., Zafeer, M.F., Ahmad, S., Akhtar, K., Husain, S.D., Ahmad, M.H., Anis, E., Hossain, M.M., 2015. In vivo induction of antioxidant response and oxidative stress associated with genotoxicity and histopathological alteration in two commercial fish species due to heavy metals exposure in northern India (Kali) river. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 176, 17–30.
- Figueira, E., Branco, D., Antunes, S.C., Gonçalves, F., Freitas, R., 2012a. Are metallothioneins equally good biomarkers of metal and oxidative stress? *Ecotoxicol. Environ. Saf.* 84, 185–190.
- Figueira, E., Cardoso, P., Freitas, R., 2012b. *Ruditapes decussatus* and *Ruditapes philippinarum* exposed to cadmium: toxicological effects and bioaccumulation patterns. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 156, 80–86.
- Findlay, J.W., Dillard, R.F., 2007. Appropriate calibration curve fitting in ligand binding assays. *AAPS J.* 9, E260–E267.
- Franzellitti, S., Buratti, S., Du, B., Haddad, S.P., Chambliss, C.K., Brooks, B.W., Fabbri, E., 2015. A multibiomarker approach to explore interactive effects of propranolol and fluoxetine in marine mussels. *Environ. Pollut.* 205, 60–69.
- Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: new data on single and

- combined long-term effects of carbamazepine on *Scrobicularia plana*. Sci. Total Environ. 541, 977–985.
- Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M., Figueira, E., 2015a. How life history influences the responses of the clam *Scrobicularia plana* to the combined impacts of carbamazepine and pH decrease. Environ. Pollut. 202, 205–214.
- Freitas, R., Almeida, Â., Pires, A., Velez, C., Calisto, V., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M., 2015b. The effects of carbamazepine on macroinvertebrate species: comparing bivalves and polychaetes biochemical responses. Water Res. 85, 137–147.
- Freitas, R., Pires, A., Quintino, V., Rodrigues, A.M., Figueira, E., 2012. Subcellular partitioning of elements and availability for trophic transfer: Comparison between the Bivalve *Cerastoderma edule* and the Polychaete *Diopatra neapolitana*. Estuar. Coast. Shelf Sci. 99, 21–30.
- Geret, F., Serafim, A., Barreira, L., Bebianno, M.J., 2002. Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam *Ruditapes decussatus*. Biomarkers 7, 242–256.
- Gonzalez-Rey, M., Mattos, J.J., Piazza, C.E., Bainy, A.C.D., Bebianno, M.J., 2014. Effects of active pharmaceutical ingredients mixtures in mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 153, 12–26.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
- Jo, P.G., Choi, Y.K., Choi, C.Y., 2008. Cloning and mRNA expression of antioxidant enzymes in the Pacific oyster, *Crassostrea gigas* in response to cadmium exposure. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 147, 460–469.
- Johansson, L.H., Borg, L.A.H., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. 174, 331–336.

- Juhel, G., Bayen, S., Goh, C., Lee, W.K., Kelly, B.C., 2017. Use of a suite of biomarkers to assess the effects of carbamazepine, bisphenol A, atrazine, and their mixtures on green mussels, *Perna viridis*. Environ. Toxicol. Chem. 36, 429–441.
- King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20, 849–854.
- Kosonen, J., Kronberg, L., 2009. The occurrence of antihistamines in sewage waters and in recipient rivers. Environ. Sci. Pollut. Res. 16, 555–564.
- La Colla, N.S., Negrin, V.L., Marcovecchio, J.E., Botté, S.E., 2015. Dissolved and particulate metals dynamics in a human impacted estuary from the SW Atlantic. Estuar. Coast. Shelf Sci. 166, 45–55.
- La Farre, M., Pérez, S., Kantiani, L., Barceló, D., 2008. Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. TrAC Trends Anal. Chem. 27, 991–1007.
- Larsson, D.J., de Pedro, C., Paxeus, N., 2007. Effluent from drug manufactures contains extremely high levels of pharmaceuticals. J. Hazard. Mater. 148, 751–755.
- Letullier, A., Minguez, L., Costil, K., Halm-Lemeille, M., Lebel, J., Serpentine, A., 2014. In vitro effect of five pharmaceuticals on the viability of the European abalone hemocytes, *Haliotis tuberculata*. J. Xenobiotics 4.
- Li, M., 2013. Acute toxicity of 30 pharmaceutically active compounds to freshwater planarians, *Dugesia japonica*. Toxicol. Environ. Chem. 95, 1157–1170.
- Li, Z., Li, P., Randak, T., 2011. Protective roles of calcium channel blocker against cadmium-induced physiological stress in freshwater teleost *Oncorhynchus mykiss*. Water. Air. Soil Pollut. 220, 293–299.

- Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. *Environ. Pollut.* 157, 561–568.
- Maffei, M., Vernocchi, P., Lanciotti, R., Guerzoni, M.E., Belletti, N., Gardini, F., 2009. Depuration of striped Venus clam (*Chamelea gallina* L.): effects on microorganisms, sand content, and mortality. *J. Food Sci.* 74, M1–M7.
- Marie, V., Gonzalez, P., Baudrimont, M., Bourdineaud, J.-P., Boudou, A., 2006. Metallothionein response to cadmium and zinc exposures compared in two freshwater bivalves, *Dreissena polymorpha* and *Corbicula fluminea*. *Biometals* 19, 399–407.
- Martin-Diaz, L., Franzellitti, S., Buratti, S., Valbonesi, P., Capuzzo, A., Fabbri, E., 2009. Effects of environmental concentrations of the antiepileptic drug carbamazepine on biomarkers and cAMP-mediated cell signaling in the mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.* 94, 177–185.
- Martins, V.V., Zanetti, M.O.B., Pitondo-Silva, A., Stehling, E.G., 2014. Aquatic environments polluted with antibiotics and heavy metals: a human health hazard. *Environ. Sci. Pollut. Res.* 21, 5873–5878.
- Metcalf, C.D., Miao, X.-S., Koenig, B.G., Struger, J., 2003. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ. Toxicol. Chem.* 22, 2881–2889.
- Meyer, J.S., Ranville, J.F., Pontasch, M., Gorsuch, J.W., Adams, W.J., 2015. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. *Environ. Toxicol. Chem.* 34, 799–808.
- Mohamed, B., Jean-Yves, M., Pierre, M., Hélène, B., Aleya, L., Elsa, B., Florence, G., 2017. Assessment of *Lemna minor* (duckweed) and *Corbicula fluminea* (freshwater clam) as potential indicators of contaminated aquatic ecosystems:

- responses to presence of psychoactive drug mixtures. Environ. Sci. Pollut. Res. 1–13.
- Monterroso, P., Abreu, S.N., Pereira, E., Vale, C., Duarte, A.C., 2003. Estimation of Cu, Cd and Hg transported by plankton from a contaminated area (Ria de Aveiro). Acta Oecologica 24, S351–S357.
- Monterroso, P., Pato, P., Pereira, M.E., Millward, G.E., Vale, C., Duarte, A., 2007. Metal-contaminated sediments in a semi-closed basin: implications for recovery. Estuar. Coast. Shelf Sci. 71, 148–158.
- Moradi, S., Nowzari, H., Farhadian, M., 2017. Assessment of cadmium and lead in the water and trout fish (*Salmo trutta*) of Zayandehroud River, a case study of Zarinshahr rice farms, Isfahan. Iran. J. Fish. Sci. 16, 188–199.
- Morin, S., Duong, T.T., Dabrin, A., Coynel, A., Herlory, O., Baudrimont, M., Delmas, F., Durrieu, G., Schäfer, J., Winterton, P., 2008. Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. Environ. Pollut. 151, 532–542.
- Nödler, K., Licha, T., Fischer, S., Wagner, B., Sauter, M., 2011. A case study on the correlation of micro-contaminants and potassium in the Leine River (Germany). Appl. Geochem. 26, 2172–2180.
- Nödler, K., Voutsas, D., Licha, T., 2014. Polar organic micropollutants in the coastal environment of different marine systems. Mar. Pollut. Bull. 85, 50–59.
- Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351–358.
- Pires, A., Almeida, Â., Calisto, V., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M., Figueira, E., Freitas, R., 2016a. *Hediste diversicolor* as bioindicator of pharmaceutical pollution: Results from single and combined exposure to carbamazepine and caffeine. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 188, 30–38.

- Pires, A., Almeida, Â., Correia, J., Calisto, V., Schneider, R.J., Esteves, V.I., Soares, A.M., Figueira, E., Freitas, R., 2016b. Long-term exposure to caffeine and carbamazepine: Impacts on the regenerative capacity of the polychaete *Diopatra neapolitana*. *Chemosphere* 146, 565–573.
- Ragusa, M.A., Costa, S., Cuttitta, A., Gianguzza, F., Nicosia, A., 2017. Coexposure to sulfamethoxazole and cadmium impairs development and attenuates transcriptional response in sea urchin embryo. *Chemosphere* 180, 275–284.
- Rahman, I., Kode, A., Biswas, S.K., 2006. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* 1, 3159–3165.
- Rittschof, D., Lai, C., Kok, L., Teo, S.L., 2003. Pharmaceuticals as antifoulants: Concept and principles. *Biofouling* 19, 207–212.
- Rittschof, D., McClellan-Green, P., 2005. Molluscs as multidisciplinary models in environment toxicology. *Mar. Pollut. Bull.* 50, 369–373.
- Robinson, H.W., Hogden, C.G., 1940. The biuret reaction in the determination of serum proteins. 1. A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. *J. Biol. Chem.* 135, 707–725.
- Rouchon, A.M., Phillips, N.E., 2017. Acute toxicity of copper, lead, zinc and their mixtures on the sea urchin *Evechinus chloroticus*. *N. Z. J. Mar. Freshw. Res.* 51, 333–355.
- Sacher, F., Lange, F.T., Brauch, H., Blankenhorn, I., 2001. Pharmaceuticals in groundwaters: analytical methods and results of a monitoring program in Baden-Württemberg, Germany. *J. Chromatogr. A* 938, 199–210.
- Spann, N., Aldridge, D.C., Griffin, J.L., Jones, O.A., 2011. Size-dependent effects of low level cadmium and zinc exposure on the metabolome of the Asian clam, *Corbicula fluminea*. *Aquat. Toxicol.* 105, 589–599.

- Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment, in: Molecular, Clinical and Environmental Toxicology. Springer, pp. 133–164.
- Teixeira, M., Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Wrona, F.J., Soares, A.M., Figueira, E., Freitas, R., 2017. Toxic effects of the antihistamine cetirizine in mussel *Mytilus galloprovincialis*. Water Res. 114, 316–326.
- Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers. Water Res. 32, 3245–3260.
- Traudt, E.M., Ranville, J.F., Smith, S.A., Meyer, J.S., 2016. A test of the additivity of acute toxicity of binary-metal mixtures of Ni with Cd, Cu, and Zn to *Daphnia magna*, using the inflection point of the concentration–response curves. Environ. Toxicol. Chem. 35, 1843–1851.
- Tsiaka, P., Tsarpali, V., Ntaikou, I., Kostopoulou, M.N., Lyberatos, G., Dailianis, S., 2013. Carbamazepine-mediated pro-oxidant effects on the unicellular marine algal species *Dunaliella tertiolecta* and the hemocytes of mussel *Mytilus galloprovincialis*. Ecotoxicology 22, 1208–1220.
- Velez, C., Figueira, E., Soares, A., Freitas, R., 2015a. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuar. Coast. Shelf Sci. 155, 114–125.
- Velez, C., Freitas, R., Antunes, S.C., Soares, A.M., Figueira, E., 2016. Clams sensitivity towards As and Hg: A comprehensive assessment of native and exotic species. Ecotoxicol. Environ. Saf. 125, 43–54.
- Velez, C., Galvão, P., Longo, R., Malm, O., Soares, A.M., Figueira, E., Freitas, R., 2015b. *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. Environ. Sci. Pollut. Res. 22, 11890–11904.

- Vicente-Martorell, J.J., Galindo-Riaño, M.D., García-Vargas, M., Granado-Castro, M.D., 2009. Bioavailability of heavy metals monitoring water, sediments and fish species from a polluted estuary. *J. Hazard. Mater.* 162, 823–836.
- Wang, L., Pan, L., Liu, N., Liu, D., Xu, C., Miao, J., 2011. Biomarkers and bioaccumulation of clam *Ruditapes philippinarum* in response to combined cadmium and benzo [α] pyrene exposure. *Food Chem. Toxicol.* 49, 3407–3417.
- Won, E., Kim, R., Rhee, J., Park, G.S., Lee, J., Shin, K., Lee, Y., Lee, J., 2011. Response of glutathione S-transferase (GST) genes to cadmium exposure in the marine pollution indicator worm, *Perinereis nuntia*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 154, 82–92.
- Wu, X., Cobbina, S.J., Mao, G., Xu, H., Zhang, Z., Yang, L., 2016. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. *Environ. Sci. Pollut. Res.* 23, 8244–8259.
- Xie, Z., Lu, G., Hou, K., Qin, D., Yan, Z., Chen, W., 2016. Bioconcentration, metabolism and effects of diphenhydramine on behavioral and biochemical markers in crucian carp (*Carassius auratus*). *Sci. Total Environ.* 544, 400–409.
- Zhao, L., Zhang, Y., Liang, J., Xu, X., Wang, H., Yang, F., Yan, X., 2014. Environmental cadmium exposure impacts physiological responses in Manila clams. *Biol. Trace Elem. Res.* 159, 241–253.

Figure Captions

Figure 1. Energy-related parameters (A: ETS, electron transport system activity; B: GLY, glycogen content; C: PROT, protein content) in *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28-day exposure period. Values are the mean \pm standard error. Significant differences ($p \leq 0.05$, $n=6$) among exposure conditions, when present, are represented with different letters.

Figure 2. Indicators of oxidative stress (A: LPO, lipid peroxidation; B: GSH/GSSG, ratio between reduced and oxidized glutathione) in *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28-day exposure period. Values are the mean \pm standard error. Significant differences ($p \leq 0.05$, $n=6$) among exposure conditions, when present, are represented with different letters.

Figure 3. Antioxidant enzyme activities (A: SOD, superoxide dismutase; B: CAT, catalase) in *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28-day exposure period. Values are the mean \pm standard error. Significant differences ($p \leq 0.05$, $n=6$) among exposure conditions, when present, are represented with different letters.

Figure 4. Biotransformation enzyme activities (GSTs, glutathione S-transferases) in *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28-day exposure period. Values are the mean \pm standard error. Significant differences ($p \leq 0.05$, $n=6$) among exposure conditions, when present, are represented with different letters.

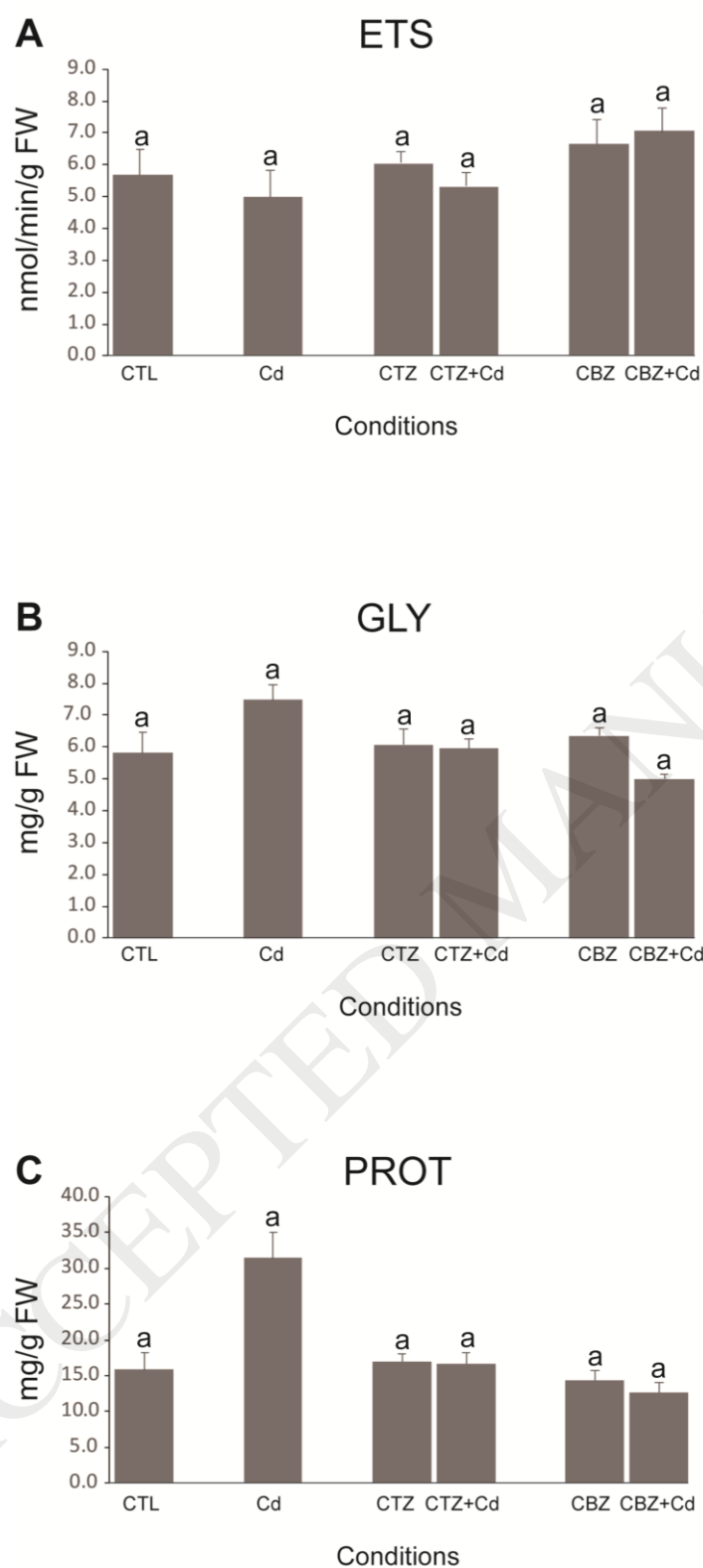
Fig 1**Figure 1**

Fig 2

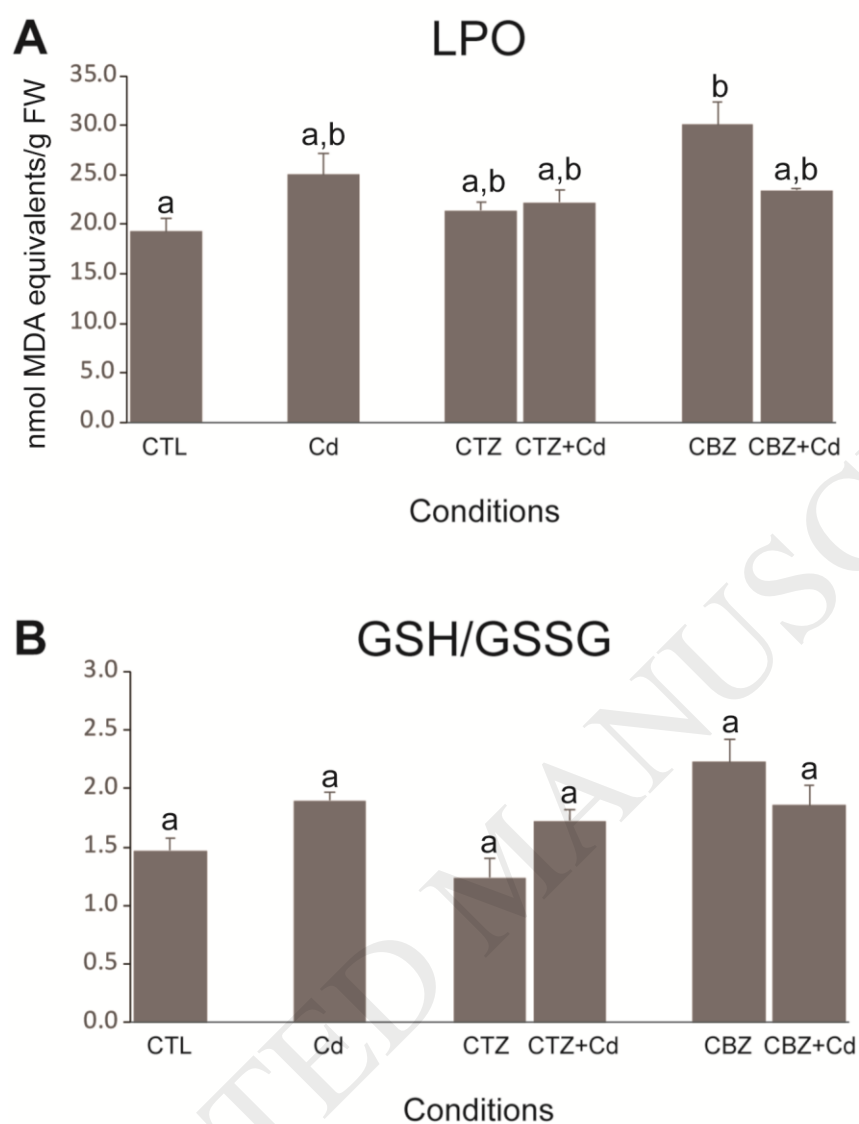


Figure 2

Fig 3

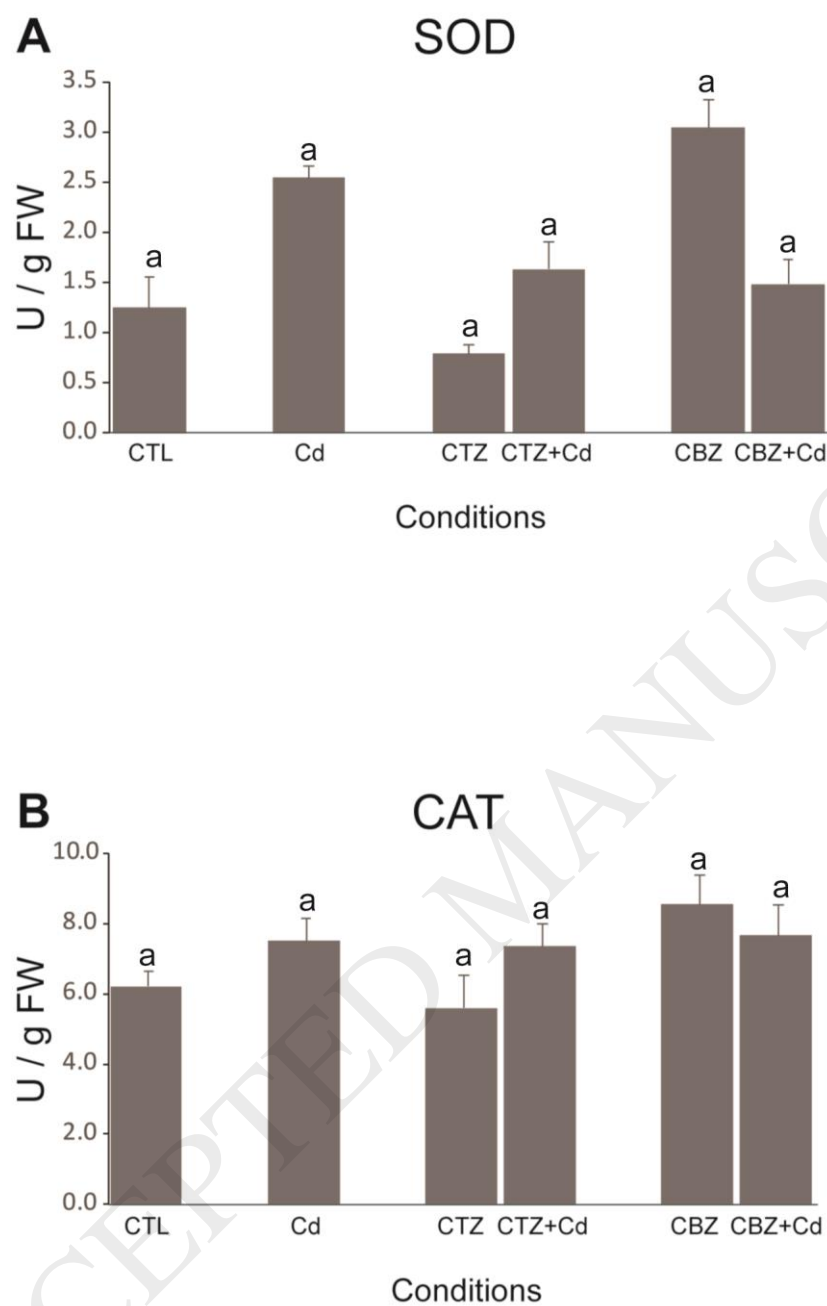
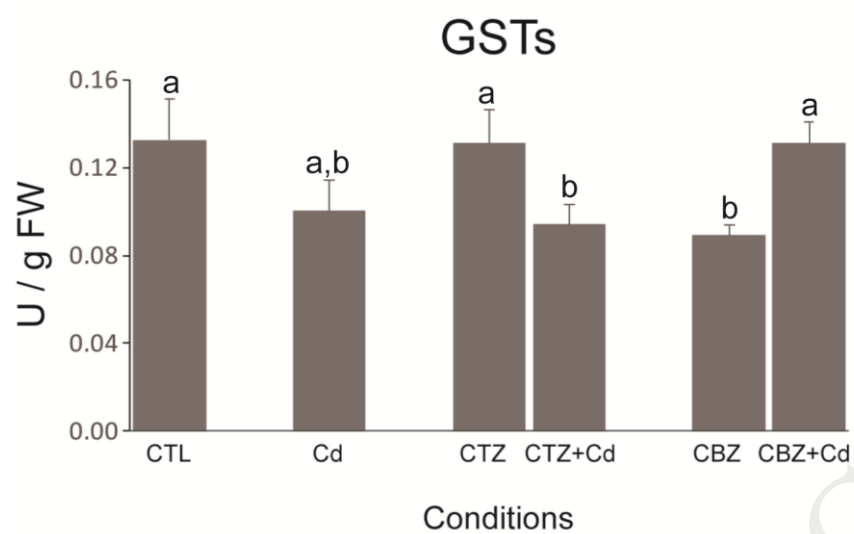


Figure 3

Fig 4**Figure 4**

Tables

Table 1. Average Cd, CBZ and CTZ concentrations ($\mu\text{g/L}$) in the exposure medium, controls and blanks of the assay conducted with *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period, with 7 days renewal periods. Values are the mean \pm STDEV. Significant differences ($p \leq 0.05$, $n=3$) among exposure conditions, when present, are represented with different letters. LOQ, limit of quantification.

Drug and metal concentration in the exposure medium and blanks ($\mu\text{g/L}$)				
Conditions	Exposure medium		Blanks	
	Beginning	End	Beginning	End
CTL	< LOQ	< LOQ	< LOQ	< LOQ
Cd	< LOQ	< LOQ	< LOQ	< LOQ
Cd (CBZ+Cd)	< LOQ	< LOQ	< LOQ	< LOQ
Cd (CTZ+Cd)	< LOQ	< LOQ	< LOQ	< LOQ
CTZ	0.45 ± 0.08^a	0.4 ± 0.1^a	0.62 ± 0.06^a	0.6 ± 0.1^a
CTZ (CTZ+Cd)	0.41 ± 0.06^a	0.4 ± 0.1^a	0.60 ± 0.09^a	0.5 ± 0.1^a
CBZ	1.1 ± 0.1^a	1.1 ± 0.1^a	1.2 ± 0.1^a	1.2 ± 0.2^a
CBZ (CBZ+Cd)	1.1 ± 0.2^a	1.1 ± 0.1^a	1.1 ± 0.2^a	0.94 ± 0.09^a

Table 2: Significance values for metal and drug quantifications in the exposure medium and blanks of the assay conducted with *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period. Significant differences ($p \leq 0.05$, $n=3$), when existing, are in bold.

Conditions	Exposure medium	Blanks
CTZ Beginning vs End	0.8387	0.7067
CTZ Beginning vs CTZ+Cd Beginning	0.2282	0.7134
CTZ+Cd Beginning vs CTZ+Cd End	0.8287	0.6493
CTZ End vs CTZ+Cd End	0.653	0.8197
CBZ Beginning vs End	0.5661	0.9445
CBZ Beginning vs CBZ+Cd Beginning	0.7196	0.107
CBZ+Cd Beginning vs CBZ+Cd End	0.4084	0.1239
CBZ End vs CBZ+Cd End	0.8864	0.4922

Table 3. Cadmium (Cd) concentrations ($\mu\text{g/g DW}$) and BCF (bioconcentration factor) in clams exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period. Cetirizine (CTZ) and carbamazepine (CBZ) concentrations (ng/g FW) and BCF in clams exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period. Values are the mean \pm STDEV. Significant differences ($p \leq 0.05$, $n=3$ for Cd samples and $n=6$ for drug samples) among exposure conditions, when present, are represented with different letters.

	CTL	Cd	CTZ	CTZ+Cd	CBZ	CBZ+Cd
Cd concentrations in clams' soft tissues ($\mu\text{g/g}$)	0.22 \pm 0.08 ^a	0.7 \pm 0.2 ^b	-	0.8 \pm 0.4 ^b	-	1.0 \pm 0.2 ^b
BCF for Cd	-	1450 \pm 370 ^a	-	2023 \pm 770 ^a	-	1560 \pm 740 ^a
Drugs concentrations in clams' soft tissues (ng/g)	-	-	2.9 \pm 0.3 ^a	3.8 \pm 0.8 ^b	2.0 \pm 0.2 ^a	1.9 \pm 0.2 ^a
BCF for drugs	-	-	4.7 \pm 0.5 ^a	6.2 \pm 1.2 ^b	1.7 \pm 0.1 ^a	1.7 \pm 0.1 ^a

Table 4. Significance values for metal and drug quantifications in the tissues and BCF (bioconcentration factor) of the assay conducted with *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period. Significant differences ($p \leq 0.05$, $n=3$ for Cd samples and $n=6$ for drug samples) are in bold.

Conditions	Comparisons	Significance values
Cd concentration in tissue	CTL vs Cd	0.0182
	CTL vs CTZ+Cd	0.0198
	CTL vs CBZ+Cd	0.0354
	Cd vs CTZ+Cd	0.369
	Cd vs CBZ+Cd	0.3206
BCF for Cd	Cd vs CTZ+Cd	0.8353
	Cd vs CBZ+Cd	0.3063
Drugs concentration in tissues	CTZ vs CTZ+Cd	0.0001
	CBZ vs CBZ+Cd	0.3741
BCF for Drugs	CTZ vs CTZ+Cd	0.0001
	CBZ vs CBZ+Cd	0.6555

Table 5. Significance values for biomarkers of the assay conducted with *R. philippinarum* exposed to different conditions (CTL, Cd, CTZ, CTZ+Cd, CBZ, CBZ+Cd), after 28 days exposure period. Significant differences ($p \leq 0.05$, $n=6$) are in bold.

	Biomarkers							
	ETS	GLY	PROT	LPO	GSH/GSSG	SOD	CAT	GSTs
CTL vs Cd	0.3977	0.6053	0.0880	0.1727	0.2086	0.1817	0.2736	0.1456
CTL vs CTZ	0.6661	0.8528	0.7557	0.6013	0.4782	0.1947	0.6685	0.9209
CTL vs CTZ+Cd	0.7824	0.9131	0.8100	0.4278	0.3584	0.5160	0.0524	0.0411
CTZ+Cd vs Cd	0.8077	0.6100	0.1057	0.1574	0.6522	0.3516	0.8734	0.7071
CTZ+Cd vs CTZ	0.5905	0.8795	0.9326	0.7270	0.2385	0.1593	0.2575	0.0446
CTL vs CBZ	0.3542	0.7088	0.5787	0.0459	0.3603	0.0928	0.1429	0.0228
CTL vs CBZ+Cd	0.2783	0.5534	0.3095	0.3884	0.2844	0.7058	0.2202	0.9115
CBZ+Cd vs Cd	0.1253	0.4270	0.0513	0.6290	0.9153	0.3056	0.9023	0.1336
CBZ+Cd	0.7547	0.2022	0.4446	⁴⁸ 0.1205	0.6571	0.1566	0.5916	0.0104

vs CBZ

ACCEPTED MANUSCRIPT