



Ecotoxicity of the antihistaminic drug cetirizine to *Ruditapes philippinarum* clams

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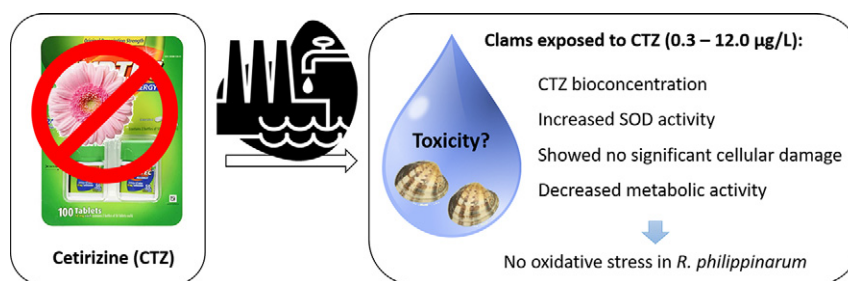
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HIGHLIGHTS

- The antihistamine cetirizine (CTZ) was bioconcentrated in clams.
- The activity of superoxide dismutase was induced.
- Absence of significant cellular damage exerted by CTZ in clams
- CTZ decreased the metabolic capacity of clams.
- No oxidative stress induced by CTZ in the clams

GRAPHICAL ABSTRACT



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ABSTRACT

Cetirizine (CTZ) is an antihistaminic drug present in the aquatic environment, with limited information on its toxicity to organisms inhabiting this system. This study intended to evaluate the effects of CTZ on oxidative stress and energy metabolism biomarkers in the edible clam *Ruditapes philippinarum* after a 28 days exposure to environmentally relevant CTZ concentrations (0.0, 0.3, 3.0, 6.0 and 12.0 µg/L). The results obtained showed that CTZ was accumulated by clams reaching maximum concentrations (up to ~22 ng/g FW) at the highest CTZ exposure concentrations (6.0 and 12.0 µg/L). The bioconcentration factor (average maximum values of ~5) decreased at 12.0 µg/L reflecting a reduction in clams uptake or increase of excretion capacity at this condition. The present study revealed that, in general, clams decreased the metabolic potential after exposure to CTZ (decrease in electron transport system activity), a response that led to the maintenance of glycogen content in organisms exposed to CTZ in comparison to control values. Our findings also showed that, CTZ did not exert significant levels of oxidative injury to clams. However, comparing the control with the highest exposure concentrations (6.0 and 12.0 µg/L) a significant increase of the antioxidant enzyme superoxide activity (~53 and ~44%) was observed in clams exposed to CTZ. Moreover, a tendency to increase lipid peroxidation (~14 and ~9%) and carbonyl groups on proteins (~11 and ~3%) was observed in clams exposed to CTZ (6.0 and 12.0 µg/L) compared to control condition. Overall the present study suggests that toxic impacts may be induced in *R. philippinarum* if exposed for longer periods or higher CTZ concentrations.

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1. Introduction

The worldwide contamination of aquatic systems by pharmaceutical drugs with toxic effects to inhabiting organisms is a well-known

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environmental issue (Monteiro and Boxall, 2010). Antihistamines are a group of pharmaceuticals commonly used for the treatment of allergic reactions and have been found in the aquatic environment due to global consumption and generally poor degradation in wastewater treatment plants (WWTPs) (Golovko et al., 2014; Kosonen and Kronberg, 2009). The highest concentrations of antihistamines in water bodies are commonly observed in spring, the season of increased concentrations of aerial pollen derived from flowering plants, which is associated to a higher consumption of these drugs. Cetirizine (CTZ), a second-generation antihistaminic drug, is a potent histamine H1 receptor antagonist with antiallergic properties. CTZ (the active substance of Zyrtek and Reactin among other pharmaceuticals) is used to treat chronic idiopathic urticaria, perennial and seasonal allergic rhinitis, allergic asthma, physical urticaria, and atopic dermatitis (DrugBank, 2017). When consumed 70% of the CTZ dose (maximum of 20 mg/tablet/day) is excreted unchanged by renal mechanisms, approximately 10% in the faeces and 8 to 10% is metabolized by the P450 cytochrome oxidase pathway (Campoli-Richards et al., 1990; DrugBank, 2017; Wood et al., 1987).

CTZ has been detected in water bodies worldwide (Bahlmann et al., 2012, 2009; Bebianno et al., 2016; Calisto et al., 2011; Fick et al., 2009; Kosonen and Kronberg, 2009; Larsson et al., 2009, 2007; Nödler et al., 2014, 2011). Kosonen and Kronberg (2009) identified the presence of antihistamines including CTZ in influents and effluents of a sewage treatment plant from Turku (Finland) with concentrations up to 220 ng/L and in recipient river waters with concentrations between 4 and 8 ng/L. These authors found that during the treatment applied in the WWTPs, the concentration of CTZ dropped about 16% of the influent level. Fick et al. (2009) found very high CTZ concentrations in an effluent of a WWTP in Patancheru (India) at 2.1 mg/L, but upstream and downstream samples showed lower CTZ concentrations, varying between 9 and 530 µg/L. CTZ was also determined in wells in India, with concentrations ranging from 550 ng/L to 28 µg/L. These authors referred that some of the tested wells were still used as drinking water sources. Bahlmann et al. (2012) reported maximum CTZ concentrations of 0.49 and 0.51 µg/L in wastewaters from, respectively, influents and effluents of Berlin (Germany) WWTPs. In surface waters, also from Berlin, CTZ was detected with concentrations up to 0.72 µg/L. Nödler et al. (2014) found CTZ in the Venice lagoon (Italy), in the San Francisco Bay (USA) and in the Baltic Sea (Germany) with maximum concentrations of 2.7, 6.3 and 13 ng/L, respectively. Recently Bebianno et al. (2016) found CTZ (up to 1.28 µg/L) in an effluent from a psychiatric hospital in Montpon (France) containing a mixture of 25 pharmaceutical drugs.

Despite the presence of CTZ in the environment, few studies evaluated the possibility of toxic risks induced by this drug to aquatic organisms, being limited to the works performed with bacteria (Bergheim et al., 2014; Borowska et al., 2016), gastropod haemocytes and larvae (Letullier et al., 2014; Rittschhof et al., 2003), bivalves adults (Teixeira et al., 2017) and plathelminthes adults (Li, 2013). Rittschhof et al. (2003) reported that CTZ (evaluated as Zyrtec) inhibited the larval settlement of the barnacle *Amphibalanus amphitrite* at 40 ng/mL. Recently, Al-Aidaros et al. (2017) also reported that CTZ, at sublethal concentrations (1/5th LC50 = 1.18 mg/L), inhibited barnacle (*Amphibalanus amphitrite*) larval development, decreased larval settlement and respiration rate, revealing the involvement of histamine in larval settlement and metamorphosis processes. Bergheim et al. (2014) studied the *in vitro* toxicity and the bio- and photopersistence of different pharmaceutical drugs, including CTZ. These authors found that CTZ (up to 6 µg/spot) was more toxic than its photoproducts in the *Vibrio fischeri* (bacteria) bioluminescence assay. However, an opposite response was observed with *Pseudomonas putida* (bacteria) growth inhibition test, with a lower percentage of inhibition for the non-irradiated drug (~10%, at 8 mg/L) comparing to the irradiated CTZ (40–60%, also at 8 mg/L). Borowska et al. (2016) tested the efficiency of wastewater ozonation for the reduction of nitrogen-containing pharmaceuticals and observed that CTZ ozonation products elicited biological effects on bacteria (*Aliivibrio fischeri*)

bioluminescence with a 10 and 50% effect concentration at 0.4 and 4 mg/L, respectively. Letullier et al. (2014) evaluated the *in vitro* effects of four pharmaceutical drugs including CTZ (0.5–500 µg/L) in haemocytes of the abalone *Haliotis tuberculata*, revealing that the drug affected lysosomal integrity, although not posing danger. Recently Teixeira et al. (2017) demonstrated that *Mytilus galloprovincialis* mussels exposed to CTZ (0.3–12.0 µg/L) increased their energy reserves and maintained metabolic potential in comparison to non-contaminated organisms. These authors also demonstrated that, although exposed mussels increased their antioxidant defenses they were not able to prevent cellular damage.

Nevertheless, besides these studies, scarce information is available regarding the impacts of CTZ in aquatic organisms induced at the cellular level, namely on biochemical alterations (Teixeira et al., 2017), which are valuable markers to provide “early-warning signals” of impairments on species performance (Colin et al., 2016). Thus, the present study aimed to evaluate the effects of CTZ on oxidative stress biomarkers and energy metabolism in the edible clam *Ruditapes philippinarum*, by exposing the organisms for 28 days to CTZ at environmentally relevant concentrations.

2. Materials and methods

2.1. Study area and test organism

The clam *R. philippinarum* (Adams & Reeve, 1850), commonly known as the Manila clam, was selected to study the ecotoxicological impact of CTZ. Clams were collected in February 2016 in the Mira channel, located in the Ria de Aveiro (Portugal) that, according to Castro et al. (2006) and Cerqueira and Pio (1999), is the least impacted channel of this ecosystem due to the less dense human settlements and to low industrial and harbour activity, which are located further North in the lagoon. The evaluation of drug contamination in the Ria de Aveiro is still scarce (Calisto et al., 2011; Paíga et al., 2013; Silva et al., 2014, 2013) but studies conducted by Calisto et al. (2011) determined the concentration of pharmaceutical drugs (the antiepileptic carbamazepine and the antihistamine CTZ) in this aquatic system, identifying CTZ in one (Costa Nova) of eleven sampling areas, with concentrations of 0.04 µg/L. The drug was also found in the wastewater influents/effluents of the Aveiro WWTPs with an average concentration of 0.3 µg/L. *R. philippinarum* is an exotic species, introduced in the Ria de Aveiro in 2010 (Maia and Gaspar, 2014), which is used in biomonitoring studies with different types of contaminants worldwide (Casatta et al., 2016; Moschino et al., 2011; Oaten et al., 2017; Piló et al., 2017; Sacchi et al., 2013; Won et al., 2016). For laboratory experiments, *R. philippinarum* individuals with similar size (mean length: 4.2 ± 0.3 cm; mean width: 3.0 ± 0.2 cm, condition index: $5.2 \pm 0.2\%$) were selected to minimize the effect of body size on biochemical response and CTZ uptake. The organisms were acclimated to laboratory conditions during 7 days in seawater (salinity 25 g/L), under continuous aeration, temperature 18 ± 1 °C and 12:12 h (light/dark) photoperiod. During the acclimation period clams were not fed.

2.2. Experimental conditions

After acclimation, clams were chronically (28 days) exposed to CTZ. The organisms were distributed by four CTZ exposure concentrations (0.3, 3.0, 6.0 and 12.0 µg/L) plus the control condition (0.0 µg/L). Each exposure concentration was prepared by spiking the medium (3 L), using a defined volume (according to the exposure concentration) of a 10 mg/L CTZ stock solution (prepared in NaCl 25 g/L). For each concentration, 3 replicates were performed, each one with 3 individuals, corresponding to a total of 9 organisms ($3 \times 3 = 9$) per condition. Organisms were placed in containers filled with 3 L of artificial seawater (salinity 25 g/L) and submitted to continuous aeration, temperature 18 ± 1 °C and a 12:12 h (light/dark) photoperiod. For each condition, blanks (two replicates) were performed to evaluate the losses of CTZ (bio-

and photodegradation or sorption onto the test vessels) during the assay. The blanks corresponded to aqueous standards of CTZ in seawater with the same concentrations used in the assay and exposed to the same conditions but without clams.

During the experiment the medium was renewed once a week and the concentrations re-established. Daily, animals were checked for mortality and fed every other day with Algamac protein plus (150,000 cells/animal). Clams were considered dead when, after external stimulus, their shells gaped and failed to shut again. At the end of exposure, the surviving organisms were frozen for further analysis.

For CTZ quantification and biochemical analysis the whole frozen soft tissues of the surviving animals were individually pulverized, in a mill, with liquid nitrogen. For each organism, the pulverized tissues were divided into aliquots of 0.3 g and preserved at -80°C until analysis. After this procedure, each aliquot was extracted with the specific buffer for each analysis (1:2, w/v). For that, samples were sonicated for 15 s at 4°C . Then, the samples were centrifuged for 20 min, at 3000g or 10,000g (depending on the biomarker) at 4°C and the supernatants were collected for CTZ quantification and for the determination of biochemical parameters. The supernatants were preserved at -80°C until analysis.

2.3. Cetirizine quantification by ELISA

2.3.1. Sample preparation

For each condition, the soft tissue aliquots (0.3 g) of the surviving clams were used for CTZ quantification. For this, clams supernatants were obtained by extraction of the pulverized soft tissues with deionized water (1:2, w/v) (Almeida et al., 2014). Also, to evaluate the behavior of CTZ in the exposure medium of each condition (0.0, 0.3, 3.0, 6.0 and $12.0\text{ }\mu\text{g/L}$), during the course of the assay (weekly), aliquots of water were collected at the beginning (immediately after the contamination spike) and at the end of each week of exposure (7 days, corresponding to the time between each water renewal). CTZ was also quantified in blanks performed to evaluate CTZ loss during the experiments.

2.3.2. Immunoassay procedure and calibration curve

CTZ was quantified based on the direct competitive ELISA (Enzyme-Linked Immunosorbent Assay), according to the procedure developed by Bahlmann et al. (2012) and Calisto et al. (2011) and the modifications described in Almeida et al. (2014) but using a sample buffer (pH 7.6) prepared with 1 M tris(hydroxymethyl)aminomethane (TRIS), 2% (w/v) ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA) and 3 M sodium chloride (NaCl). 96-well high-binding microtiter plates were coated with a polyclonal anti-mouse antibody overnight. After this incubation, the plates were washed and the monoclonal antibody against CTZ was incubated for 1 h. After this time, another washing step was performed and the tracer solution, the standards and samples were incubated for 30 min. After another washing step, the substrate solution was added to each well and the plate incubated for 30 min. The enzyme reaction was stopped by the addition of 1 M sulphuric acid. The absorbance was measured on a microplate spectrophotometer at 450 nm and referenced to 650 nm. All samples and standards ($0\text{--}100\text{ }\mu\text{g/L}$) were determined in triplicate on each plate. A four-parametric logistic equation (4PL) (Findlay and Dillard, 2007) was fitted to the standards mean absorbance values. For the analysis of clams tissues, the standards were prepared in ultrapure water by diluting a 10 mg/L stock solution of CTZ (also prepared in ultrapure water). For the analysis of water samples, the standards were prepared by diluting the stock solution of CTZ in 25 g/L NaCl.

2.4. Biomarker measurements

The evaluation of the toxic effects induced by CTZ in *R. philippinarum* clams was done by measuring oxidative stress and energy-related

biomarkers. These biomarkers proved to be rapid and sensitive tools that give important information on the sub-lethal drug effects (Almeida et al., 2015, 2014). The soft tissue aliquots (0.3 g) of the surviving clams were used for biochemical measurements. Supernatants were used to determine: energy reserves (GLY, glycogen); metabolic capacity (ETS; electron transport system activity); indicators of cellular damage (LPO, lipid peroxidation; CP, carbonyl groups on proteins); antioxidant enzymes activity (SOD, superoxide dismutase; CAT, catalase; GPx, total glutathione peroxidase) and biotransformation enzymes activity (GSTs, glutathione S-transferases). All biochemical parameters were performed twice. For LPO, supernatants were extracted using 20% (w/v) trichloroacetic acid (TCA). For GLY, CP, CAT, SOD, total GPx and GSTs, the extraction was done with potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM EDTA; 1% (v/v) Triton X-100; 1% (w/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT); pH 7.0). For ETS determination, supernatants were extracted in 0.1 M Tris-HCl pH 8.5, 15% (w/v) PVP, $153\text{ }\mu\text{M}$ magnesium sulfate (MgSO_4) and 0.2% (v/v) Triton X-100.

2.4.1. Energy-related parameters

The quantification of GLY was done according to the sulphuric acid method (Dubois et al., 1956), using glucose standards ($0\text{--}2\text{ mg/mL}$). Absorbance was measured at 492 nm. The concentration of GLY was expressed in mg per g of fresh weight (FW).

ETS activity was measured based on De Coen and Janssen (1997) and King and Packard (1975) method. The absorbance was measured at 490 nm. 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.

2.4.2. Indicators of cellular damage

LPO levels were measured based on the Ohkawa et al. (1979) method. Absorbance was measured at 535 nm ($\epsilon = 156\text{ mM}^{-1}\text{ cm}^{-1}$). LPO was expressed in nmol of MDA formed per g FW.

CP levels were determined following the methodology described by Mesquita et al. (2014) and Levine et al. (2000). Absorbance was measured at 450 nm ($\epsilon = 22,308\text{ M}^{-1}\text{ cm}^{-1}$). CP content was expressed in nmol per g FW.

2.4.3. Antioxidant enzymes

The activity of SOD was quantified based on the Beauchamp and Fridovich (1971) method, with modifications (Carregosa et al., 2014). Standards of SOD were prepared ($0.25\text{--}60\text{ U/mL}$). SOD activity was measured spectrophotometrically at 560 nm and expressed in U per g FW ($\text{U} = \mu\text{mol/min}$).

CAT activity was quantified according to Johansson and Borg (1988) and modifications performed by Carregosa et al. (2014), using formaldehyde standards ($0\text{--}150\text{ }\mu\text{M}$). CAT activity was measured at 540 nm and expressed in U per g FW ($\text{U} = \text{nmol/min}$).

The activity of total GPx was determined following the Paglia and Valentine (1967) method. GPx activity was measured spectrophotometrically at 340 nm ($\epsilon = 0.00622\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$) and expressed in U per g FW ($\text{U} = \text{nmol/min}$).

2.4.4. Biotransformation enzymes

The activity of GSTs was determined according to Habig et al. (1974) and modifications described by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at 340 nm ($\epsilon = 9.6\text{ mM}^{-1}\text{ cm}^{-1}$). The enzymatic activity was expressed in U per g FW ($\text{U} = \mu\text{mol/min}$).

2.5. Data analysis

The bioconcentration factor (BCF) for each condition, was determined by dividing the concentration of CTZ present in clams tissue by

an average of the CTZ concentrations in the exposure medium over the entire exposure (Gobas and Morrison, 2000).

Hypothesis testing was performed by Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001). All data analysis was performed with PRIMERv6 software (Clarke and Gorley, 2006) with the PERMANOVA + add-on suite (Anderson et al., 2008). For each variable (biomarkers and CTZ determinations), the test statistic (pseudo-F test) in the main test was determined. To run the PERMANOVA tests we considered 9999 Monte Carlo permutations. The main test was evaluated in terms of significance and when significant ($p \leq 0.05$), pairwise comparisons were performed using a fixed factor. When p values in the pairwise test were lower than 0.05, the null hypothesis (described below) was rejected, being considered as significantly different. Values of $p \leq 0.05$ revealed that groups differed significantly, and significant differences were presented in figures with different letters. Significant differences between exposure periods (beginning vs end), only in the case of the exposure medium and blanks, were depicted with asterisks. To test the hypothesis that no significant differences exist in CTZ concentration (in the exposure medium and blanks) over the exposure period duration (7 days), pairwise comparisons were done, for each exposure concentration, between the beginning and end of the exposure period. To test the hypothesis that no significant differences exist in *R. philippinarum* biomarkers and CTZ bioconcentration among conditions, pairwise comparisons were done among exposure concentrations (0.0, 0.3, 3.0, 6.0 and 12.0 $\mu\text{g/L}$).

A matrix gathering the biomarker responses and the CTZ tissue concentration per condition (0.0, 0.3, 3.0, 6.0 and 12.0 $\mu\text{g/L}$) was used to calculate an Euclidean distance similarities matrix. The data used to calculate the matrix were previously normalised and transformed (square root). The matrix was then simplified through the calculation of the distance among centroid based on condition and then submitted to ordination analysis, performed by Principal Coordinates (PCO). Spearman correlation vectors ($r > 0.75$) of biochemical responses and CTZ concentration in clam tissues were superimposed on the PCO graph.

3. Results

3.1. Mortality

Only one dead clam (11%) was found at CTZ 0.3 $\mu\text{g/L}$ during the experiment. In the remaining conditions, no mortality was observed.

3.2. Cetirizine concentration in the exposure medium and in clams

For each condition tested, the concentrations of CTZ in aliquots of exposure media (Fig. 1A) collected immediately after the contamination spike (day 0) were similar to the nominal concentrations (0.3: 0.51 ± 0.05 $\mu\text{g/L}$; 3.0: 3.7 ± 0.4 $\mu\text{g/L}$; 6.0: 7.0 ± 0.7 $\mu\text{g/L}$; 12.0: 12.9 ± 0.6 $\mu\text{g/L}$). Comparing the beginning and the end of the exposure period (7 days, the period between water renewals), at each condition, a significant decrease was observed. It is important to notice that the concentrations between the beginning and the end of four weeks of exposure followed the same pattern and for this reason only the fourth week of exposure was chosen as model to show the results of CTZ in the exposure medium.

The concentration of CTZ in blanks (Fig. 1B), for each tested concentration (0.3, 3.0, 6.0 and 12.0 $\mu\text{g/L}$), remained stable comparing both sampling periods (beginning and end of 7 days exposure period) and significantly increased with the increase of exposure concentrations. As for the results of CTZ determination in the exposure medium, only the fourth week of exposure was chosen to represent the blanks since the concentrations between the beginning and the end of the remaining weeks remained similar.

CTZ concentrations in *R. philippinarum* tissue (Fig. 2A) increased significantly after exposure to each tested concentration (0.3, 3.0, 6.0 and 12.0 $\mu\text{g/L}$), comparing with control condition. The highest CTZ uptake

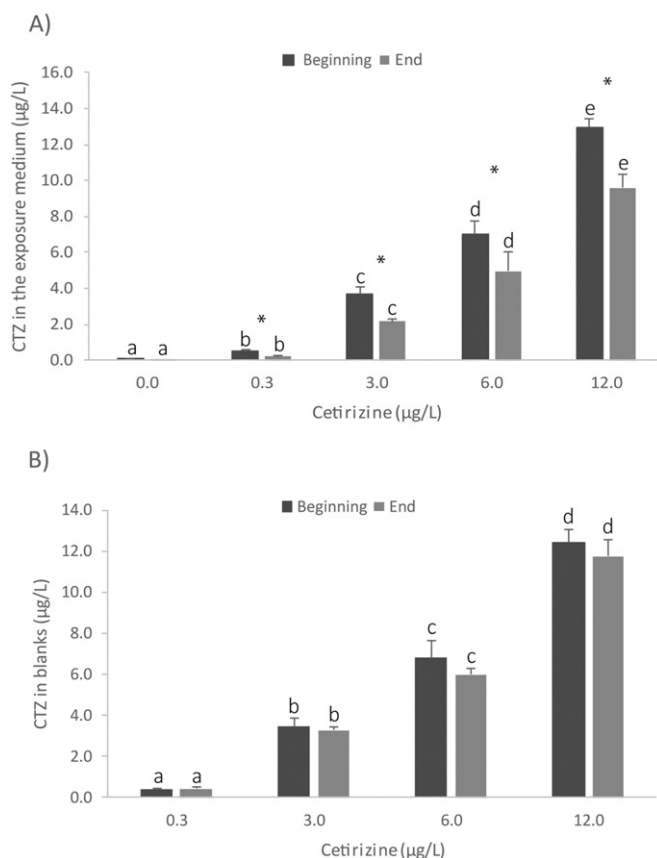


Fig. 1. CTZ concentration ($\mu\text{g/L}$) in exposure medium (A) and blanks (B). For each tested concentration, samples were collected at the beginning and the end of the fourth week of exposure. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters. Significant differences ($p \leq 0.05$) between exposure periods for each concentration are represented by an asterisk.

(~ 22 ng/g FW) was observed at 6.0 and 12.0 $\mu\text{g/L}$, with no significant differences between these two conditions.

The BCF significantly decreased comparing the highest tested concentration (12.0 $\mu\text{g/L}$) and the remaining concentrations (0.3, 3.0 and 6.0 $\mu\text{g/L}$), which were similar between each other.

3.3. Biomarker responses

3.3.1. Energy-related parameters

GLY content (Fig. 3A) did not differ significantly in clams exposed to different conditions, although a decrease was observed in organisms exposed to 3.0 $\mu\text{g/L}$ comparing with 6.0 and 12.0 $\mu\text{g/L}$.

The ETS activity (Fig. 3B) significantly decreased in clams after exposure to CTZ 0.3, 3.0 and 12.0 $\mu\text{g/L}$ concentrations by comparison with the control condition. At 6.0 $\mu\text{g/L}$ the ETS activity was similar to all the remaining exposure conditions (Fig. 3B).

3.3.2. Indicators of cellular damage

Results obtained concerning LPO are depicted in Fig. 4A. Despite the absence of significant differences among conditions, a tendency to an increase of LPO was recorded in clams exposed to the highest concentrations (6.0 and 12.0 $\mu\text{g/L}$).

The content in CP (Fig. 4B) was not significantly different among conditions. However, a tendency to an increase at 6.0 $\mu\text{g/L}$ and 12.0 $\mu\text{g/L}$ was observed by comparison with the remaining concentrations.

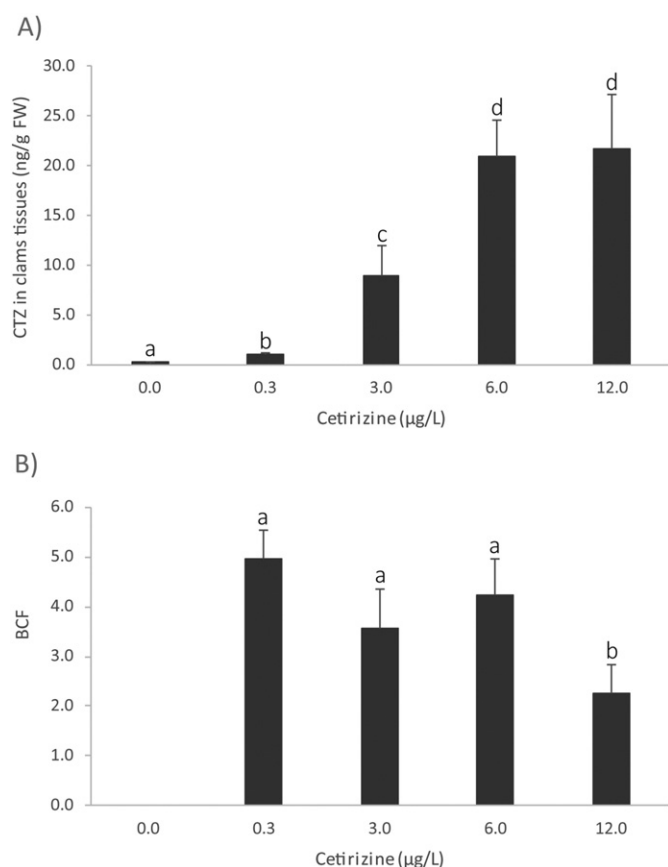


Fig. 2. CTZ concentration in clams tissues (ng/g FW) (A) and bioconcentration factor (BCF) (B) in *R. philippinarum* exposed to increasing concentrations of CTZ for 28 days. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters.

3.3.3. Antioxidant enzymes

The activity of SOD (Fig. 5A) increased at CTZ 6.0 and 12.0 µg/L, by comparison with the remaining conditions (control, 0.3 and 3.0 µg/L) but significant differences were only found between these two conditions and 3.0 µg/L. At CTZ 0.3 and 3.0 µg/L SOD activity was similar to control.

Regardless the condition tested, the activity of CAT (Fig. 5B) did not show significant differences in clams exposed to different CTZ concentrations. However, a tendency to a decrease in CAT activity was observed comparing clams exposed to CTZ (0.3, 3.0, 6.0 and 12.0 µg/L) with control.

The activity of total GPx decreased significantly in clams exposed to 0.3, 3.0 and 6.0 µg/L CTZ in comparison to control condition, while at 12.0 µg/L no significant differences were observed in comparison to control values (Fig. 5C).

3.3.4. Biotransformation enzymes

The activity of GSTs (Fig. 6) showed no significant differences among tested conditions.

3.4. Multivariate analysis of the biomarker responses and CTZ concentration in clams

The centroid PCO ordination graph, resulting from multivariate analysis of data concerning biochemical measurements and CTZ tissue concentrations for each condition tested (CTL = 0.0, 0.3, 3.0, 6.0 and 12.0 µg/L) is represented in Fig. 7. The PCO axis 1 explained 59.2% of the total variation, separating the lower concentrations (0.0, 0.3 and

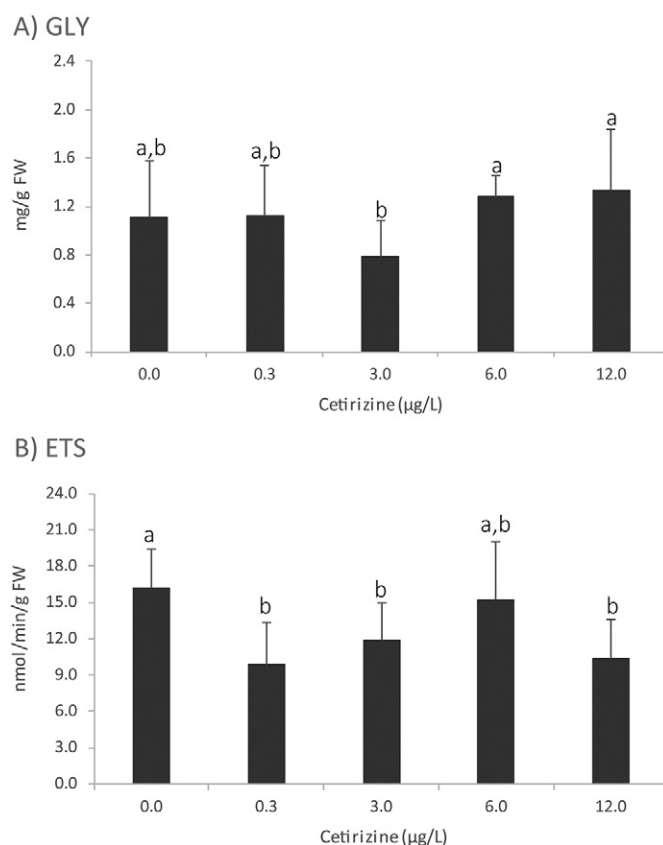


Fig. 3. Energy-related parameters (A: GLY, glycogen; B: ETS, electron transport system activity) in *R. philippinarum* exposed to increasing concentrations of CTZ during 28 days. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters.

3.0 µg/L) on the positive side of the axis, from the highest concentrations (6.0 and 12.0 µg/L) on the negative side of the axis. This axis was better explained by higher CTZ concentrations ($r = -1$). The negative side of this axis, with the highest CTZ concentrations, was also correlated with LPO ($r = -0.7$) and CP ($r = -0.6$) levels and SOD ($r = -0.6$) activity. The PCO axis 2 explained 27.7% of the total variation, dividing the control and 6.0 µg/L on the positive side of the axis, from 0.3, 3.0 and 12.0 µg/L conditions on the negative side of the axis. Control condition, on the axis 2 positive side, was closely related to CAT activity ($r = 0.7$), where higher values were recorded.

4. Discussion

Cetirizine (CTZ) has been reported in aquatic environments, but few studies evaluated the toxic effects exerted by this drug on aquatic organisms, especially under chronic exposure and using environmentally relevant concentrations. Except for mussels (Teixeira et al., 2017), previous studies, mainly studied acute exposures performed with high concentrations (up to 8 mg/L), reported that CTZ does not pose toxicity to aquatic organisms or, the damage occurs only at non-environmental concentrations. However, the toxicity of CTZ to aquatic organisms, in particular invertebrates, may be underestimated due the scarce information available on this issue. Thus, the present study aimed to provide insights, using energy-related and oxidative stress biomarkers, on the effects of CTZ in the edible clam *R. philippinarum*, after 28 days of exposure to CTZ environmental concentrations (0.0, 0.3, 3.0, 6.0 and 12.0 µg/L).

Concerning CTZ behavior in the exposure medium and blanks our results showed that, at the beginning of the exposure period, the spiked concentrations were in general similar to the nominal concentrations, validating the spiking methodology and confirming that the clams

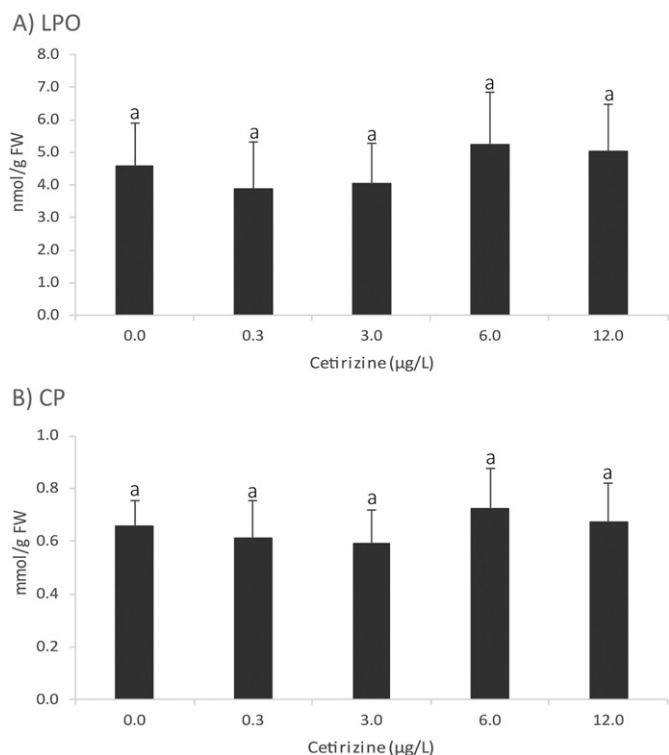


Fig. 4. Indicators of cellular damage (A: LPO, lipid peroxidation; B: CP, carbonyl groups on proteins) in *R. philippinarum* exposed to increasing concentrations of CTZ during 28 days. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters.

were exposed to statically different CTZ levels. After 7 days of exposure CTZ concentrations in the exposure medium decreased at all conditions (by comparison with the beginning of exposure). However, the same decrease was not observed in blanks, in which the concentrations remained stable over the exposure period (7 days), indicating that CTZ was not adsorbed onto the surface of the exposure vessels neither degraded (bio- and photodegradation). Thus, the present study indicates an interaction between the drug and clams (e.g. accumulation), revealing an uptake of the drug up to 22 ng per g FW (at 6.0 and 12.0 µg/L). However, at 12.0 µg/L the decrease in BCF (on average 50% in comparison with the remaining CTZ concentrations) may indicate a restrain in the uptake or excretion capacity at this condition. The reduction in drug uptake was already observed in bivalves exposed to other pharmaceutical drugs, and was justified as a decrease in the uptake due the reduction in filter feeding activity (Almeida et al., 2015, 2014; Chen et al., 2014; Freitas et al., 2016; Matozzo et al., 2003). Almeida et al. (2015) explained the decrease in the BCF for *R. philippinarum* exposed at the highest concentration of antiepileptic carbamazepine (9.0 µg/L, 28 days of exposure) as a decrease in the clearance rate by clams. The capacity of aquatic organisms to metabolize and excrete pharmaceutical drugs was also reported in literature (Contardo-Jara et al., 2011; Garcia et al., 2012). Nevertheless, despite low BCF levels (average maximum of ~5) obtained in the present study, which demonstrate the low potential for CTZ bioconcentration, the results show that accumulation of this antihistamine by aquatic organisms is possible.

When assessing biochemical alterations induced in invertebrates by pollutants, energy-related biomarkers as glycogen (GLY) content and electron transport system (ETS) activity have been applied (Smolders et al., 2004, 2003). GLY is an important energy reserve in bivalves (Hummel et al., 1989). Lagadic et al. (1994) reported that under stress an increase or a decrease in glycogenolysis (glycogen breakdown) can occur but, in most cases, depletion of the GLY reserves is observed due to increased energy demand. On the other hand, Freitas et al. (2015a) related the maintenance of GLY levels in the clam *Scrobicularia plana*

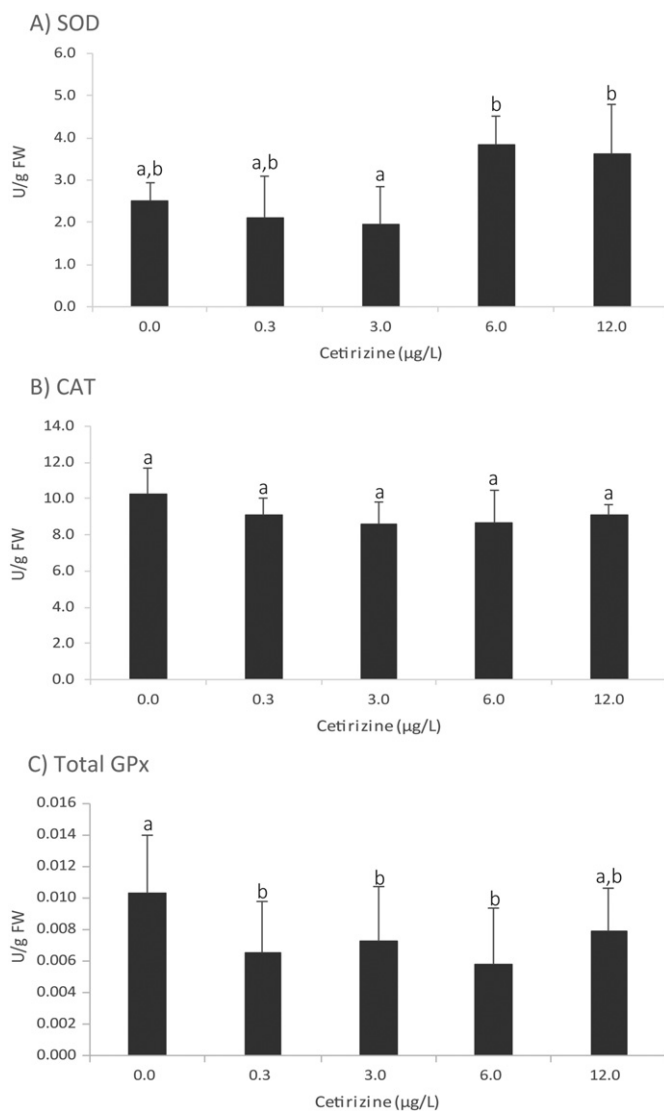


Fig. 5. Antioxidant enzymes (A: SOD, superoxide dismutase; B: CAT, catalase; C: GPx, total glutathione peroxidase) in *R. philippinarum* exposed to increasing concentrations of CTZ during 28 days. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters.

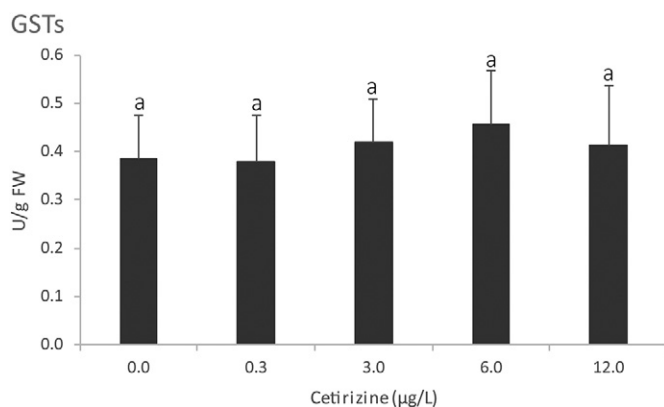


Fig. 6. Biotransformation enzymes (GSTs, glutathione S-transferases) in *R. philippinarum* exposed to increasing concentrations of CTZ during 28 days. Values are the mean and standard deviation. Significant differences ($p \leq 0.05$) among exposure concentrations are represented with different letters.

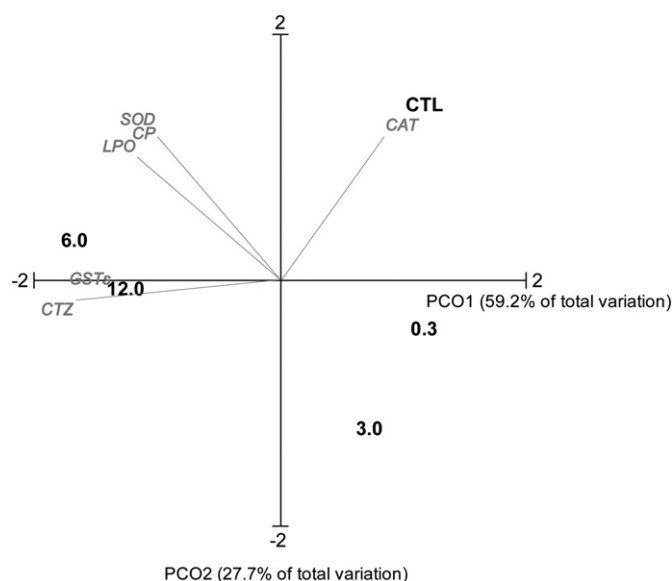


Fig. 7. Principal Coordinates (PCO) graph based on biomarker responses and CTZ tissue concentrations (CTZ) in *R. philippinarum* exposed to increasing concentrations of CTZ during 28 days. The control (CTL) and the concentrations (0.3, 3.0, 6.0 and 12.0 µg/L) are indicated. The biomarkers presented are: CAT, catalase; SOD, superoxide dismutase; LPO, lipid peroxidation; CP, carbonyl groups on proteins and GSTs, glutathione S-transferases.

exposed to CBZ with the reduction in ETS activity, thus a lower metabolic potential. In this way, the balance between ETS activity and the energy reserves as GLY has been used as biomarkers related to the energy metabolism in invertebrates (De Coen and Janssen, 1997; Smolders et al., 2004). The present study revealed that clams presented lower metabolic potential after exposure to CTZ (decrease in ETS), except at 6.0 µg/L, a response that led to the maintenance of GLY content in organisms exposed to CTZ in comparison to control values, as observed by Freitas et al. (2015a).

Oxidative stress biomarkers have been providing valuable information to understand, quickly and easily, if organisms are suffering oxidative damage. Previous studies showed changes in oxidative stress biomarkers after exposure, among others, to pharmaceutical drugs such as the antiepileptic carbamazepine (Almeida et al., 2015, 2014, Freitas et al., 2016, 2015a, 2015b; Martin-Diaz et al., 2009); the non-steroidal anti-inflammatory drugs acetaminophen, diclofenac, ibuprofen, ketoprofen and nimesulfide (Brandão et al., 2014; Mezzelani et al., 2016); the selective serotonin reuptake inhibitor fluoxetine (Di Poi et al., 2016); the psychoactive drug caffeine (Cruz et al., 2016); and the antihistaminic cetirizine (Teixeira et al., 2017). Oxidative stress occurs due to an imbalance between the production of reactive oxygen species (ROS) and their elimination by the antioxidant system defense. When ROS are in excess, protein degradation and enzyme inactivation, DNA damage and lipid peroxidation occur (Regoli and Winston, 1999). Lipid peroxidation (LPO) levels observed in the present study showed that CTZ did not significantly induced oxidative damage in membrane lipids. Although our study showed no cellular damage induced by CTZ in *R. philippinarum* clams, Teixeira et al. (2017) demonstrated that CTZ (0.3–12 µg/L) clearly induced LPO in *Mytilus galloprovincialis* mussels after 28 days of exposure. These findings may suggest higher tolerance of clams to CTZ in comparison to mussels. Also, other antihistamine drugs led to LPO increase in aquatic organisms. Xie et al. (2016) reported that diphenhydramine (at 21.7 and 112 µg/L), a second-generation antihistamine, led to the increase in LPO in the fish *Carassius auratus* after 7 days of exposure. However, it is important to note that the concentrations of exposure used by Xie et al. (2016) were higher than the ones applied in the present study, which may justify results concerning LPO obtained by the referred authors. Moreover, unlike CTZ,

diphenhydramine is a second-generation antihistamine having adverse side effects as for example, a cholinergic activity.

Another consequence of oxidative stress injury is the formation of carbonyl groups in proteins (CP). The carbonyl groups are formed on protein side chains, under oxidative stress, thus changing the proteins structure, function and integrity (Dalle-Donne et al., 2003). Our findings revealed no significant increase of CP content, supporting the hypothesis that the CTZ concentrations tested are not capable of inducing significant levels of oxidative stress in *R. philippinarum*. Although the studies in the literature showed the induction of oxidative damage by antihistamines to aquatic organisms (Teixeira et al., 2017; Xie et al., 2016), few studies were performed, which reinforces the necessity of conducting more studies to better evaluate the impacts of antihistamines to aquatic organisms.

The antioxidant enzymes are used by cells to eliminate ROS and maintain oxidative status (Colin et al., 2016; Regoli and Giuliani, 2014; Regoli and Principato, 1995). Among antioxidant enzymes, superoxide dismutase (SOD) converts superoxide anion to hydrogen peroxide; catalase (CAT) converts hydrogen peroxide to water and total glutathione peroxidase (GPx) that detoxifies hydrogen peroxide or organic hydro-peroxides produced by lipid peroxidation (Regoli and Principato, 1995). Our findings showed that SOD and CAT responses agreed with results on LPO and CP results, revealing that organisms did not significantly present increased activity of antioxidant enzymes at lower CTZ concentrations since there was no evidence of oxidative injury. Indeed, a tendency to a decrease in CAT activity was observed after exposure to the drug at the lower concentrations (mainly at 0.3 and 3.0 µg/L), possibly reflecting a beneficial effect of the presence of CTZ such a reduction in oxidation levels. This reduction is possibly related with the mode of action of antihistamines. For example, Lojek et al. (2011) showed the inhibitory effects of some antihistaminic drugs in ROS generation by macrophages involved in inflammatory reactions. At CTZ 6.0 and 12.0 µg/L, however, increased SOD activity, possibly to mitigate the observed tendency of LPO and CP increase, suggesting that this enzyme was effective in ROS elimination, avoiding a significant level of oxidative damage (LPO and CP). On the contrary, when evaluating the impacts of CTZ in mussels, Teixeira et al. (2017) showed that although CTZ induced the activity of antioxidant enzymes (SOD and CAT) mussels were not able to prevent cellular damage observed through the increase of LPO. Such findings may indicate that CTZ induce higher toxicity in mussels (*M. galloprovincialis*) in comparison to clams (*R. philippinarum*). Different response in SOD and CAT activities were also reported in literature after exposure of *R. philippinarum* to pharmaceutical drugs. Almeida et al. (2014) observed an increase in SOD activity in clams exposed to the highest CBZ exposure concentration (9 µg/L) for 96 h, although not being capable to eliminate the ROS induced by the presence of CBZ, thus triggering the induction of LPO. Milan et al. (2013) evaluated the effects of the ibuprofen (0–1000 µg/L), a non-steroidal anti-inflammatory drug, in *R. philippinarum* after 1, 3, 5 and 7 days of exposure. These authors observed that after 7 days of exposure to ibuprofen, SOD activity decreased in the clams digestive gland comparing with control condition, suggesting that the drug reduced the antioxidant defenses activity. Matozzo et al. (2012) observed no significant differences in CAT activity in the gills and digestive gland of *R. philippinarum* exposed to triclosan for 7 days (300–900 ng/L), an antimicrobial and antifungal agent present in personal care products. Accordingly, these authors found no significant changes in LPO after exposing clams to triclosan. Cruz et al. (2016) showed an increase in CAT activity after the exposure of *R. philippinarum* to caffeine (3 and 18 µg/L) for 28 days. These authors reported that although CAT activity increased with the increase of caffeine concentrations, this enzymatic defense was not able to eliminate the excessive ROS responsible for the increase in LPO. Such findings may indicate that CTZ may be less toxic to *R. philippinarum* than other drugs. Regarding GPx activity, a decrease in the enzymatic activity after exposure to CTZ (on average 33%) was observed in the present study. Since the drug did not exert

oxidative damage, the decrease in the GPx enzymatic activity, as for CAT activity, possibly reflect a beneficial effect of the presence of CTZ, somehow contributing to the decrease of oxidative stress, especially at 0.3, 3.0 and 6.0 µg/L. However, other works found a different explanation for the decrease in GPx observed in their studies. For example, a decrease in the GPx activity was found by Aguirre-Martínez et al. (2016) after exposing *R. philippinarum* clams to the antiepileptic carbamazepine and to the antibiotic novobiocin (0.1–50 µg/L) for 14 days. However, these authors justified the decrease as being a consequence of the oxidative stress enhanced by the exposure of clams to prooxidant drugs. In the present case, it seemed that the hydrogen peroxide produced by the increased SOD activity was not enough to increase the activity of GPx and CAT, neither to significantly increase LPO. Other studies demonstrated an increase in GPx related with the increase of hydrogen peroxide levels resulting from the exposure to pharmaceutical drugs. Also, Xie et al. (2016) found an increase in GPx activity in the liver of the fish *Carassius auratus* exposed for 7 days to the antihistamine diphenhydramine (0.84–112 µg/L). The authors justified the increase in GPx activity based on the increase of hydrogen peroxide, despite SOD activity was decreased. Xie et al. (2016) referred that SOD activity was inhibited by the excessive production of ROS that possible damaged SOD protein, however that was still in the GPx elimination capacity.

Besides the antioxidant system defense, organisms have biotransformation enzymes, involved in the detoxification of xenobiotics. Among these, glutathione S- transferases (GSTs) are a family of enzymes of phase II drug-metabolism that catalyse the conjugation of reduced glutathione with electrophilic compounds. This process transforms the xenobiotics into more water-soluble compounds to improve their excretion rate by the organisms (Penner et al., 2012). GSTs are also involved in the inactivation of endogenous secondary metabolites formed during oxidative stress as hydro-peroxides resulting from lipid peroxidation (Penner et al., 2012). In the present study, the results concerning GSTs activity showed no significant involvement in CTZ biotransformation, which are in accordance with Teixeira et al. (2017) findings with mussels. Literature available for humans already demonstrated that CTZ is poorly metabolized (El-Kommos et al., 2015; Wood et al., 1987). Xie et al. (2016) found a decrease in GSTs activity after exposing *C. auratus* to the antihistamine diphenhydramine (0.84–112 µg/L) for 7 days, and related these results to ROS formation (due to CYP induction) and the incapacity to conjugate the antihistamine through the glutathione pathway.

5. Conclusions

The chronic toxic effects of environmental concentrations of the antihistamine CTZ to *R. philippinarum* clams were investigated through the study of energy-related and oxidative stress biomarkers. At the lowest exposure concentrations studied (0.3 and 3.0 µg/L) CTZ was not harmful to clams, with no evidence of oxidative stress and metabolic alterations. Although at a limited extent, the presence of CTZ in clam tissues seemed to induce oxidative stress at 6.0 and 12.0 µg/L, with significant increase of SOD activity observed at these conditions (increase of ~52% and 44% respectively, in comparison to control). This antioxidant defense appeared to be effective in eliminating the stress, avoiding significant oxidative damage (no significant changes in LPO). Thus, the results of the present study showed that CTZ at environmentally relevant concentrations did not exert significant levels of oxidative stress to *R. philippinarum* clams. Despite that, the results concerning LPO and CP (tendency to increase) may indicate that longer exposure to the drug, as occurs in the environment, and exposure to higher concentrations can possible lead to toxic impacts in clams. Since *R. philippinarum* is one of the most economically relevant bivalve species in the world (Chiesa et al., 2017), if the damage occurs the clams survival capacity can be affected, eliciting not only ecological damages but also negative economic impacts.

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