Chronic toxicity of the antiepileptic carbamazepine on the clam

Ruditapes philippinarum

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1. Introduction

Pharmaceuticals are used throughout the world being not only applied in human medicine but also in livestock breeding and aquaculture (Heberer et al., 2002; Boxall et al., 2004). Many of the pharmaceutical drugs applied in human/animal medicine are not completely metabolized by the organism, being excreted as a mixture of the unchanged compound, metabolites (active or inactive) or conjugates (mostly glucuronides and sulfates) (Daughton and Ternes, 1999; Heberer et al., 2002). Once excreted, these drugs reach the environment directly or via Wastewater Treatment Plants (WWTPs), where the majority end up passing unaltered through the conventional removal treatments applied (Monteiro and Boxall, 2010). As a consequence, the drugs are released in the environment possibly reaching concentrations that will trigger toxic effects on non-target organisms. If the organisms possess receptors interacting with the drug effects may arise on them (Gunnarsson et al., 2008). Bivalves, due to their filter feeding and respiration capacities (McEniff et al., 2014), are among the most threatened organisms to the presence of contaminants in the water column (Gagné et al., 2006a). The uptake of pharmaceutical residues in wild bivalves has been previously shown (Klosterhaus et al., 2013; Martínez-Bueno et al., 2013), and trace concentrations of drugs were already detected in cooked seafood (McEniff et al., 2013). However, few studies addressed the effects of those contaminants on these organisms (e.g. Gagné et al., 2006a; Canesi et al., 2007; Parolini and Binelli, 2012), being necessary additional information to better understand the toxicity that drugs may pose to this group of marine invertebrates.

Among pharmaceutical drugs commonly detected in the aquatic environment, carbamazepine (CBZ) is the most representative compound belonging to the class of antiepileptic pharmaceuticals (Mohapatra et al., 2014). This drug has antiepileptic and psychotropic properties, exerting its effects by blocking the sodium channels of excitatory neurons (Malavízhi et al., 2012). The high consumption and the low degradation rate upon WWTPs (~10%) (Zhang et al., 2008) are the principal reasons for the occurrence of CBZ in water bodies, namely in WWTP...
influenst and effluents, surface waters, groundwater and even in treated drinking water, with concentrations ranging from 0.03 to 6.3 μg/L (Ternes, 1998; Sacher et al., 2001; Ferrari et al., 2003; Metcalfe et al., 2003; Bahlmann et al., 2009, 2012; Calisto et al., 2011a).

Although a vast literature is available about the quantification of pharmaceutical drugs in environmental samples, there is still a long way to go through regarding their toxic impacts, especially concerning chronic exposures, since the majority of works looking at the biological effects of pharmaceuticals have thus far concentrated on acute studies (Quinn et al., 2008). Furthermore, since CBZ is a persistent drug when released into the environment, requiring between 4.5 and 25 sunny summer days for its elimination (Calisto et al., 2011b), the evaluation of a long-term exposure is necessary to fully understand the impact of persistent drugs on aquatic organisms. Studies focusing on acute toxicity found that, in general, the CBZ concentrations causing effects occur in the mg/L range (Malarvizhi et al., 2012), which are not representative of those concentrations occurring in the aquatic ecosystem, thus, leading to conclude that the risk of acute toxic effects in the environment is unlikely (Fent et al., 2006). However, considering that pharmaceutical drugs are continuously discharged into aquatic media, the assessment of chronic toxicity is of utmost importance. Furthermore, aquatic organisms downstream WWTP effluents are chronically exposed to a complex mixture of synthetic and biologically active pharmaceuticals which may impair important biological processes (Liu et al., 2015).

Ferrari et al. (2003) studied the acute and chronic effects of CBZ, on the microcrustacean Ceriodaphnia dubia, observing that the chronic tests displayed higher toxicity than the acute assays. Other authors found a similar pattern, where the chronic tests presented a higher toxicity than the acute tests (e.g. Oetken et al., 2005; Zhang et al., 2012). Moreover, chronic studies performed with environmental concentrations have shown that CBZ can affect multiple oxidative and biochemical pathways in bivalves, with significant deleterious effects (Martin-Diaz et al., 2009; Contardo-Jara et al., 2011; Chen et al., 2014). The impact of CBZ was reported in other species, namely algae (Tsiaka et al., 2013), crustacean (Aguirre-Martinez et al., 2013a) and fish (Gagné et al., 2006a; Li et al., 2009, 2010).

Despite the literature concerning CBZ toxicity, the impact of CBZ on aquatic organisms is still poorly studied (Chen et al., 2014). The present study aimed to determine the effects of a chronic exposure (28 days) to environmentally relevant concentrations of CBZ (0.00-9.00 μg/L) in the clam Ruditapes philippinarum. The cost-effective immunoassay ELISA (Enzyme-Linked ImmunoSorbent Assay) was used for CBZ quantification in the bivalve's homogenates and the toxicity of CBZ was evaluated by the analysis of a battery of physiological and biochemical parameters.

2. Materials and methods

2.1. Study area and test organism

The Ria de Aveiro is a shallow estuary located in the Northwest Atlantic coast of Portugal, which comprises a complex net of channels and wide intertidal areas (mudflats and salt marshes) (Jonkers et al., 2010). This aquatic system has three main channels radiating from the sea entrance, named Mira, São Jacinto and Ilhavo. The lagoon has been impacted due to the low industrial and harbor activity and to the less dense human settlements. Freitas et al. (2014) pointed out that Mira channel is relatively pristine due to the low metal and metalloid concentrations. For these reasons, the Mira channel was chosen as the sampling area for this study.

R. philippinarum (Adams & Reeve, 1850), commonly known as the Manila clam, was selected to study the ecotoxicological impact of CBZ in the Ria de Aveiro. This species has already been used in previous studies, under laboratory and field conditions, to assess the toxicity of CBZ (Aguirre-Martinez et al., 2013a, 2013b; Almeida et al., 2014) as well as other pharmaceutical drugs (Contardo-Jara et al., 2011; Matozzo et al., 2012a; Antunes et al., 2013; Milan et al., 2013) and to assess organic and metal pollution (Riba et al., 2004; Martin-Diaz et al., 2007; Figueira et al., 2012). All these studies demonstrated the suitability of R. philippinarum as a bioindicator species in marine environment quality assessment.

For laboratory experiments 90 individuals with similar size (mean length: 4.17 ± 0.3 cm; mean width: 3.22 ± 0.4 cm) were selected in order to minimize the effect of body weight on biochemical response and CBZ uptake. After sampling, organisms were transported to the laboratory where they were deprived during 8 days in seawater (salinity 25.0 g/L), under continuous aeration, constant temperature of 18 ± 1 °C and a photoperiod of 12:12 h (light/dark). The clams were fed with Algalac protein plus (150,000 cells/animal) every other day.

2.2. Experimental conditions — toxicity tests

The chronic toxicity assay lasted 28 days and the organisms of R. philippinarum were distributed by five concentrations of CBZ (control = 0.00; 0.03; 0.30; 3.00 and 9.00 μg/L). The tested concentration range of CBZ was selected based on concentrations found in the environment and at the Ria de Aveiro (0.03 to 6.3 μg/L; Bahlmann et al., 2009, 2012; Calisto et al., 2011a; Ferrari et al., 2003; Metcalfe et al., 2003; Sacher et al., 2001; Ternes, 1998). The highest concentration (9.00 μg/L) was selected to simulate an increase in CBZ discharges into the aquatic environment. For each concentration 18 individuals were used, placed each one in a different plastic container. Each container was filled with 300 mL of medium (artificial seawater, salinity 25.0 g/L), and submitted to continuous aeration, a temperature of 18 ± 1 °C and a photoperiod of 12:12 h (light/dark). Daily, animals were checked for mortality and every other day they were fed with Algalac protein plus (150,000 cells/animal). Clams were considered dead when their shells gaped and failed to shut again after external stimulus. During the experiment, the water was renewed twice a week and the concentrations re-established. At the end of exposure, the surviving organisms were frozen for further analysis.

2.3. Physiological parameters

2.3.1. Condition index

The condition index (CI) was determined according to Matozzo et al. (2012b) using 5 clams per conditions. CI values were calculated corresponding to the percentage of the ratio between the dry weight of the soft tissues (g) and the dry weight of the shell (g).

2.3.2. Clearance rate

The clearance rate (CR) was determined according to Coughlan (1969), based on the absorption rate of a neutral red dye by clams (Masilamoni et al., 2002). Following the chronic exposure, 5 clams per condition were contaminated with a Congo Red solution (15 mg/L). After observing the valve aperture and during 5 h with intervals of 1 h, the dye concentration in aliquots of 1 mL of the solution was measured spectrophotometrically at 498 nm. The clearance rate (L/h) was calculated using the equation (Coughlan, 1969):

\[ CR = \frac{V}{mt} \times \log(C_i/C_f) \]
were previously demonstrated using ELISA to quantify CBZ in R. philippinarum homogenate extracts, with no matrix interferences and no sample pre-treatment, turning the assay suitable for large and economic environmental screenings (Almeida et al., 2014). Good recoveries were previously demonstrated using ELISA to quantify CBZ in R. philippinarum homogenate extracts, with no matrix interferences and with no sample pre-treatment, turning the assay suitable for large and economic environmental screenings (Almeida et al., 2014). This assay is based on a direct competition of the antigen (CBZ) and tracer (CBZ analogue linked to the enzyme horseradish peroxidase) for the primary antibody (monoclonal antibody against CBZ) binding sites that are immobilized in a 96-well microtiter plate via a secondary antibody. High-binding microtiter plates were coated with this polyclonal antibody against mouse IgG (1 mg/mL per well) diluted in phosphate buffered saline (PBS) (10 mM sodium dihydrogen phosphate, 70 mM sodium hydrogen phosphate, 145 mM sodium chloride, pH 7.6). The plates were covered with Parafilm® to prevent evaporation and incubated overnight (approximately 16 to 18 h) on a Titramax 100 plate shaker at 900 rpm. After overnight incubation, the plates were washed three times with PBS containing 0.05% (w/v) Tween™ 20 (PBS-T) using an automatic 8-channel plate washer. The monoclonal antibody against carbamazepine was also diluted in PBS (7.61 × 10^{-5} mg/mL, 200 μL per well), added and incubated for 1 h. Then, a new three-cycle washing step of the plate was performed. Afterwards, 50 μL of tracer solution (147 pmol/L in sample buffer) and 150 μL of CBZ standard solutions or samples were added per well and incubated for 30 min. The sample buffer consisted of 1 M glycine, 3 M sodium chloride and 2% (w/v) of EDTA, pH 9.5. After another three-cycle washing step (PBS-T), 200 μL of substrate solution was added per well and incubated for 30 min. The substrate solution consisted of 540 μL TMB-based solution (41 mM 3,3′,5,5′-tetramethylbenzidine (TMB), 8 mM tetrabutylammonium borohydride (TBAH) prepared in dimethylacetamide (DMA) under nitrogen atmosphere) in 21.5 mL of substrate buffer (220 mM citric acid, 0.66 mM sorbic acid potassium salt and 3 mM hydrogen peroxide). The TMB solution was freshly prepared for each run. The enzyme reaction was stopped by the addition of 1 M sulfuric acid (100 μL per well) and the optical density was read on a microplate spectrophotometer at 450 nm and referenced to 650 nm. Data was analyzed using SoftMax® Pro Software (version 5.3, Molecular Devices). All samples and standards were determined in triplicate on each plate. A four-parameter logistic equation (4PL) (Findlay and Dillard, 2007) was fitted to the standards' mean values.

A calibration curve was performed for each plate using eight calibrators, with concentrations between 0 and 100 μg/L for a better convergence of the curve fitting. For the analysis of the clams’ supernatants, the standards were prepared, in ultrapure water, by diluting a 10 mg/L stock solution of CBZ (also prepared in ultrapure water). For the analysis of water samples, the standards were prepared in seawater (25 g/L NaCl) by diluting a stock solution of CBZ of the same concentration in order to minimize the effects of the water salinity in the CBZ quantification.

2.5. Biomarker measurements

For the biomarker measurements the supernatants obtained from the pulverized soft tissues (0.5 g), prepared as referred before, were extracted with the specific buffer for each analysis (1:2, w/v). For that, the samples were sonicated for 15 s at 4 °C and centrifuged for 10 min at 10,000 g at 4 °C. Supernatants were used to determine: glycogen (GLYC) content, protein (PROT) content, electron transport system (ETS) activity, lipid peroxidation (LPO), reduced (GSH) and oxidized (GSSG) glutathione content, superoxide dismutase (SOD) activity, catalase (CAT) activity, glutathione S-transfereases (GSTs) activity and cytochrome P450 3A4 (CYP3A4) activity. All the biochemical parameters were determined twice, using the supernatants obtained for each one of five replicates (clams). For LPO, supernatants were extracted using 20% (w/v) trichloroacetic acid (TCA). For CAT, SOD, GSTs, GLYC and PROT content, the extraction was done with sodium phosphate buffer (50 mM sodium dihydrogen phosphate monohydrate; 50 mM disodium hydrogen phosphate dihydrate; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (v/v) polyvinylpyrrolidone (PVP); 1 mM diethylethritol (DDT)). For ETS determination, supernatants were extracted in homogenizing buffer (0.1 M Tris–HCl pH 8.5, 15% (w/v) PVP, 153 mM magnesium sulfate (MgSO₄) and 0.2% (v/v) Triton X-100. GSH and GSSG were determined 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). CYP3A4 activity were analyzed in supernatants extracted with sodium phosphate buffer, pH 7.4 (0.1 M sodium dihydrogen phosphate monohydrate; 0.15 M potassium chloride (KCl); 1 mM EDTA; 1 mM DTT).

2.5.1. Energy-related parameters

Glycogen (GLYC) was quantified according to the sulfuric acid method (Dubois et al., 1956), using glucose standards (0–2 mg/mL). Absorbance was measured at 492 nm. The concentration of glycogen was expressed in mg per g of fresh weight (FW).

Protein (PROT) content was determined following the spectrophotometric method of Biuret (Robinson and Hogden, 1940), using bovine serum albumin (BSA) as standard (0–40 mg/mL). Absorbance was read at 540 nm. Results were expressed in mg per g of FW.

The electron transport system (ETS) activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). The absorbance was read at 490 nm during 10 min with intervals of 25 s. The amount of formazan formed was calculated using ε = 15,900 M⁻¹ cm⁻¹ and the results expressed in nmol/min/g FW.

2.5.2. Indicators of oxidative stress

Lipid peroxidation (LPO) was measured according to Ohkawa et al. (1979) and modifications referred by Carregosa et al. (2014). Absorbance was read at 535 nm (ε = 156 mM⁻¹ cm⁻¹). LPO levels were expressed in nmol of MDA formed per g of FW.

Reduced (GSH) and oxidized (GSSG) glutathione content were determined according to Rahman et al. (2007), using reduced and oxidized glutathione standards (0–60 μmol/L). Absorbance was measured...
at 412 nm. The results were expressed as nmol per g of FW. The GSH/GSSG ratio was determined.

2.5.3. Antioxidant enzymes

Superoxide dismutase (SOD) activity was determined based on the method of Beauchamp and Fridovich (1971), with some adaptations (Carregosa et al., 2014). Standards of SOD were prepared (0.25–60 U/mL). SOD activity was measured spectrophotometrically at 560 nm. The enzymatic activity was expressed in U/g of FW.

Catalase (CAT) activity was quantified according Johansson and Borg (1988) and modifications performed by Carregosa et al. (2014). Standards of formaldehyde (0–150 μM) were prepared. Absorbance was read at 540 nm. CAT activity was expressed in U/g of FW.

2.5.4. Biotransformation enzymes

The activity of glutathione S-transferases (GSTs) was determined according to Habig et al. (1974) and the modifications described by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at 340 nm (ε = 9.6 mM·cm⁻¹). The enzymatic activity was expressed in U/g of FW.

The activity of cytochrome P450 3A4 (CYP3A4) was determined according to Habig et al. (1974) and modifications performed by Carregosa et al. (2014). Standards of benzyl ether (BzRes) were used (Quinn et al., 2004). The signal from resorufin formation was read after the incubation with 5 mM nicotinamide adenine dinucleotide phosphate (NADPH) by fluorometry (excitation wavelength 530 nm, emission wavelength 585 nm). Enzyme activity was expressed in U/g of FW.

2.6. Data analysis

The Bioconcentration Factor (BCF) was determined dividing the concentration of CBZ present in clams’ tissues by the spiked CBZ concentration for each exposure condition (Gobas and Morrison, 2000).

Physiological and biochemical descriptors and CBZ concentration data were submitted to hypothesis testing using permutation multivariate analysis of variance with the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). A one-way hierarchical design, with CBZ exposure concentration as the main fixed factor, was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. When the main test revealed statistical significance, pairwise comparisons were performed. The t-statistics in the pairwise comparisons were evaluated in terms of significance. Values lower than 0.05 were considered as significantly different. The null hypothesis tested was: no significant differences exist among exposure concentrations. Significant differences among exposure concentrations are presented with letters (a–c).

3. Results

3.1. Mortality

At the end of the exposure (28 days) mortality was observed at the concentrations of 0.03 (5%), 0.30 (5%) and 3.00 μg/L (11%). At the control condition and 9.00 μg/L no mortality was recorded.

3.2. Physiological parameters

Regarding the condition index (CI) no significant differences were found across concentrations (Fig. 1A).

The results obtained for clearance rate (CR), presented in Fig. 1B, showed a significant decrease when R. philippinarum was exposed to CBZ 9.00 μg/L comparing with the remaining conditions. For lower CBZ concentrations the filtration rates were similar (0.30 and 3.00 μg/L) or significantly higher (0.03 μg/L) than the values found at the control.

3.3. Carbamazepine quantification by ELISA

The results for CBZ quantification in R. philippinarum tissues revealed an increase in concentration along the exposure gradient (Table 1). Table 1 also presents the Bioconcentration Factor (BCF), showing a
3.4.1. Energy-related parameters

Protein (PROT) content showed a decreasing trend along the CBZ gradient, with significant differences at CBZ 0.30 and 9.00 μg/L (Fig. 2B).

The electron transport system (ETS) activity results (Fig. 2C) revealed a decreasing tendency after the exposure to CBZ. However, significant differences were only obtained between the control and the highest CBZ concentration.

3.4.2. Indicators of oxidative stress

R. philippinarum chronically exposed to CBZ exhibited lower LPO levels than the control, exception made for 3.00 μg/L, where no significant differences were found compared to the control condition (Fig. 3A).

Reduced (GSH) and oxidized (GSSG) glutathione contents (Fig. 3B) differed between conditions: higher GSH and GSSG levels were found at CBZ 3.00 and 9.00 μg/L, comparing with the remaining conditions; a significant decrease in GSH/GSSG (Fig. 3C) at the higher concentrations (0.30, 3.00 and 9.00 μg/L) by comparison with the control and 0.03 μg/L was noticed.

3.4.3. Antioxidant enzymes

Superoxide dismutase (SOD) activity depicted in Fig. 4A, showed a significant increase at all exposure concentrations compared to the control, except at CBZ 9.00 μg/L where no significant differences were found.

The activity of catalase (CAT) (Fig. 4B) showed no significant differences across all conditions, except at CBZ 0.03 μg/L where the activity of the enzyme was significantly lower compared to the control.

3.4.4. Biotransformation enzymes

The results for glutathione S-transferases (GSTs) are presented in Fig. 5A, showing no significant differences among conditions except at CBZ 3.00 μg/L, where a significant increase in the activity of this family of enzymes was observed.

The activity of the cytochrome P450 3A4 (CYP3A4) was significantly induced after exposure to CBZ, especially noticed at the lower concentrations (0.03 and 0.30 μg/L) (Fig. 5B), decreasing at the higher CBZ concentrations (3.00 and 9.00 μg/L), although being still higher than the control.

3.5. Multivariate analysis regarding clams’ biochemical responses

In Fig. 6 the centroid PCO ordination graph is represented resulting from applying a multivariate analysis to the physiological and biochemical parameters. The PCO axis 1 explained 40.2% of the total variation of data, separating the control at the negative side of the axis; 0.03 and 0.30 μg/L near the origin of the axis and 3.00 and 9.00 μg/L at the positive side. The PCO axis 2 explained 32.5% of the total data variation, separating the lower CBZ concentrations 0.03 and 0.30 μg/L from applying a multivariate analysis to the physiological and biochemical parameters of CBZ during 28 days. Values are the mean (STDEV) of five replicates. Significant differences (p ≤ 0.05) among exposure concentrations are presented with letters (a–c).
Discussion

The toxic impact of CBZ has been assessed, with the majority of studies focusing on acute effects. These studies revealed that, in general, the concentrations causing toxic effects occur in the mg/L range, which turns the risk of acute toxicity in the environment unlikely to occur. Other works pointed out the necessity to conduct more chronic tests since, in the environment, the organisms are exposed for long periods to pharmaceutical drugs. Recent studies assessing the chronic toxicity of drugs have been showing higher toxicity than the acute tests, revealing that aquatic organisms of different species (e.g. algae, crustaceans, fish) can be impaired even when exposed to environmentally relevant concentrations of drugs (Martin-Diaz et al., 2009; Li et al., 2010; Aguirre-Martínez et al., 2013a; Tsiaka et al., 2013; Chen et al., 2014).

The ability of organisms to accumulate CBZ (under laboratory or field conditions) has been reported in recent studies (Ramirez et al., 2007; Vernouillet et al., 2010; Contardo-Jara et al., 2011; Garcia et al., 2012; Martínez-Bueno et al., 2013). CBZ is relatively hydrophobic, enabling the capacity to partition into the lipid portion of the organisms and bioaccumulate (Liu et al., 2015). In the present study, CBZ in clam tissues increased with the increase of exposure concentration reaching a maximum of ≈6 ng/g FW at 9.00 μg/L. Contardo-Jara et al. (2011) determined the accumulation of CBZ in the mussel *Dreissena polymorpha* after 1, 4 and 7 days of exposure, when submitted to concentrations ranging from 0.236 to 236 μg/L and an increase in the CBZ accumulation with the exposure time was found at all concentrations, demonstrating the risk of CBZ bioaccumulation in wildlife populations.

Regarding the Bioconcentration Factor (BCF), determined in this study, the higher values were found at CBZ 0.03 and 3.00 μg/L, being approximately 1 and the lowest (≈0.7) at CBZ 9.00 μg/L. In the study performed by Contardo-Jara et al. (2011) the highest BCF values were found at the lowest CBZ concentrations where the mussels exposed to 0.236 μg/L accumulated CBZ 17-fold within 1 day. The BCF increased to 60 and 90 after 4 and 7 days of exposure, respectively. Garcia et al. (2012) compared the BCF (laboratory experiment) and BAF (field organisms) for CBZ in fish (*Pimephales notatus, Ictalurus punctatus, Oreochromis niloticus*) and found that BCF values ranged between 1.5 and 7.1, while BAFs ranged from 2.5 to 3.8, suggesting that the accumulation of CBZ under laboratory and field conditions are similar. The
The use of biochemical parameters were observed. Thus, energy-related parameters in toxicological studies is relevant since they allow the measuring of the energy costs that a fight to a stress response entails.

The lower values observed for BCF in the present study (~0.7) may be justified by the results obtained for clearance rate (CR). CR values indicated that at the lowest CBZ concentrations, *R. philippinarum* has a filter activity similar to the control condition. However, at 9.00 μg/L a significant decrease in CR was observed, in comparison with the remaining conditions. At the highest concentration, this drug may be somehow signalized as harmful, being the rate of its uptake reduced as an attempt to limit the exposure to CBZ contamination. This defense behavior may explain the lowest mortality recorded at the highest CBZ concentration (0%, 9.00 μg/L) compared to the remaining exposure concentrations (between 5 and 11%, 0.03–3.00 μg/L). Gosling (2003) reported that bivalves can isolate their tissues from the external environment by closing their valves thus, protecting themselves against contaminants. The closure of valves in the presence of CBZ was also observed by Chen et al. (2014) when submitting the freshwater clam *Corbicula fluminea* to 5 and 50 μg/L of CBZ during 30 days, indicating that the exposure to environmentally relevant concentrations is enough to alter the siphoning behavior in these clams. With this strategy, that seems to be used as a last resort, and that was observed only at the highest concentration (9.00 μg/L) the clams try to mitigate the deleterious effects that an excessive accumulation of CBZ would trigger; however, the tested lowest concentrations appear not to be high enough to activate this mechanism. Indeed, at the lowest concentrations several changes on different biochemical parameters were observed. Thus, energy-related parameters, like glycogen (GLYC) or protein (PROT) content, were applied as indicators of environmental stress (Smolders et al., 2004). The use of these parameters in toxicological studies is relevant since they allow the measuring of the energy costs that a fight to a stress response entails.

The results obtained in the present study may indicate that at CBZ 9.00 μg/L the reduction of CR could result in a lower metabolic activity, thus, the internal storages as GLYC content would not decrease, as reported by Duquesne et al. (2004). These authors observed that the reduction in clearance rate in the bivalve *Macoma balthica*, as an attempt to limit the exposure to contamination, led to a slowdown in metabolism resulting in a relatively stable GLYC concentration. Under stress PROT content can either increase or decrease. Smolders et al. (2003) reported that low to intermediate levels of pollution trigger increased protein synthesis (e.g. metabolic defenses involved in detoxification processes), but decreased protein synthesis was also referred, being attributed to high concentrations of pollutants (Smolders et al., 2004), when organisms cannot handle oxidative stress. In the current work PROT content showed a decreasing trend along the CBZ gradient. These results may indicate that the protein budget is being depressed by the exposure to CBZ, especially at the highest CBZ concentrations.

The balance between mitochondrial electron transport system (ETS) activity and energy reserves (sugars, lipids and proteins) has been reported as a relevant biochemical/physiological marker to predict changes in invertebrates (De Coen and Janssen, 1997; Smolders et al., 2004; Gagné et al., 2006c). In the present study the control condition presented the highest ETS activity, which suggests a higher metabolic rate leading to a higher expense of energy stores, as observed by the lowest GLYC content at this condition. ETS activity decreased along the exposure gradient which may reflect the reduction in CR and the slowdown of metabolism thus, mobilizing less energy stores, as GLYC.

The assessment of CBZ ecotoxicological impact revealed that the oxidative stress induced by CBZ in aquatic organisms is related to its accumulation (Quinn et al., 2008; Vernouillet et al., 2010; Contardo-Jara et al., 2011; Tsuika et al., 2013). In the present study, LPO levels at the control condition were similar (11 ± 2 nmol MDA/g FW) to those found in clams collected from the environment. These results support
the ETS activity at the control condition because a high ETS is related to a high generation of ROS (reactive oxygen species), since mitochondria are the principal site of their production. An increase in LPO levels occurred along CBZ 0.03, 0.30 and 3.00 μg/L, but decreased at CBZ 9.00 μg/L. At the highest CBZ concentrations, the ETS activity was significantly lower when compared to the remaining conditions, thus generating a lower content of ROS in mitochondria. But, the lower activity of the cytochrome P450 3A4 (CYP3A4), at this condition also accounted for the LPO decrease. This enzyme is involved in the biotransformation of CBZ, with generation of ROS as by-products of the metabolism process. In the present work, at the lowest CBZ concentrations (0.03, 0.30 μg/L) a higher CYP3A4 activity was observed, evidencing a higher capacity to metabolize CBZ and a low intracellular accumulation of the drug. However, this metabolism capacity was decreased in the presence of higher CBZ concentrations (3.00 and 9.00 μg/L), possibly revealing that the high levels of the drug inhibit CYP3A4 activity, resulting in a higher cellular accumulation of CBZ. Antioxidant enzyme activity counteracts the damaging effects of ROS formed during the metabolism of CBZ. In this study, the higher SOD activity at the lower CBZ concentrations is in accordance with the results obtained for CYP3A4 since its activity is increased at the lower CBZ concentrations (0.03, 0.30 μg/L). This increased enzymatic activity enhanced the elimination of ROS which were formed either by the metabolism of CBZ or by the ETS activity, thus conducting to a reduction in LPO levels, as observed for 0.03 and 0.30 μg/L. At CBZ 3.00 μg/L, SOD also seemed to be actively involved in the ROS elimination, but at CBZ 9.00 μg/L the enzymatic activity decreased, consequently increasing oxidative stress and LPO levels that did not reach higher values, due to the lower ETS activity. In the studies performed by Li et al. (2009) SOD activity decreased along the concentration gradient of CBZ (0.1 μg/L, 0.2 and 2 μg/L) after a 21 day exposure of the rainbow trout, Oncorhynchus mykiss. After a prolonged exposure (42 days), a strong inhibition of SOD activity was observed and attributed to the overproduction of ROS and the relatively low activity of the antioxidant system.

The activity of catalase (CAT) is related with SOD activity, since CAT decomposes the hydrogen peroxide produced by SOD into water and oxygen. However, in the present study, the CAT activity did not change significantly after the exposure to CBZ. Similarly, Martin-Diaz et al. (2009) reported no significant alteration on CAT activity in the mussel Mytilus galloprovincialis exposed to CBZ (10 μg/L, 7 days). On the other hand, Chen et al. (2014) observed that the increase in CAT activity after exposure of the clam C. flumenae to CBZ (5 and 50 μg/L, 30 days) was due to the hydrogen peroxide formation resulting from the increase in superoxide anion production and its conversion by SOD. Our findings may indicate that the hydrogen peroxide produced by SOD is possibly being converted not by CAT but by another enzyme with the same role, as glutathione peroxidase (GPx).

Besides cytochrome P450 enzymes, GSTs are another family of biotransformation enzymes involved in drug metabolism. These enzymes are involved in the formation of thiol metabolites resulting from CBZ oxidation, in which GSH is conjugated with the carbonyl group of CBZ (Vernouillet et al., 2010). GST isoenzymes are also capable of inactivating lipoperoxidation products, such as lipid hydroperoxides (Sturve et al., 2008) by the use of GSH as a reducing agent that is oxidized to GSSG when peroxides are reduced (Contardo-Jara et al., 2010). In the present study, the activity of GSTs was only significantly increased at CBZ 3.00 μg/L, in comparison with the remaining conditions, although a 20% increase of GST activity was noticed for the control and at 9.00 μg/L compared to 0.03 and 3.00 μg/L. Despite the increase in GSTs activity at CBZ 3.00 μg/L, possibly indicating an involvement in CBZ biotransformation, it seemed not to be very effective in the scavenging of the reactive metabolites, as the LPO levels were high at this condition. However, the high ROS concentrations might be due to intracellular CBZ increase and ETS activity. The increase of GSH at CBZ 3.00 and 9.00 μg/L, one of the most important scavengers of ROS, may also indicate the high need of cells to neutralize ROS. GSH/GSSG ratio decreased at the higher CBZ concentrations (0.30, 3.00 and 9.00 μg/L) being indicative of the high oxidative status occurring at these conditions.

5. Conclusions

It is generally accepted that the presence of pharmaceutical drugs in the aquatic environment poses a risk to wildlife. Although it is unlikely that these contaminants will be found at concentrations high enough to illicit an acute effect, evidence is growing, suggesting that they may be present in concentrations high enough to cause chronic effects. Despite the effort that is being made to obtain more chronic and realistic data, there is yet a large gap concerning this issue. In this way, this work was performed in order to better understand the chronic effects of CBZ, a ubiquitous pharmaceutical drug, in the aquatic environment.

Overall, the results obtained pointed out a dose-dependent effect. The control condition was described by higher GSH/GSSG ratio and ETS activity. Although presenting high LPO levels this was considered a "normal status" for the clams at this condition, revealing their high metabolic rate. The clams exposed to the lower CBZ concentrations (0.03 and 0.30 μg/L) were well correlated with a high CBZ biotransformation, indicated by the high activity of CYP3A4 and the low CBZ accumulation. Part of the ROS formed during this step was eliminated by SOD. Nevertheless, the antioxidant system defenses seemed to be properly activated at these conditions. Finally, the clams exposed to the higher CBZ concentrations (3.00 and 9.00 μg/L) were well correlated to LPO and GSSG data, indicating the higher oxidative environment at these conditions that possibly compromised the activity of SOD, CYP3A4 and GSTs (at CBZ 9.00 μg/L). Moreover, the GLYC content was the parameter with the larger variation relative to CBZ 9.00 μg/L, possibly revealing the metabolism slowdown. At this condition, the induction of GSH and the decrease of ETS were the only strategies to mitigate the oxidative damage, resulting from the high CBZ accumulation and the low enzymatic activity.

A prolonged exposure to CBZ leads to a strong inhibition of antioxidant enzymes, compromising the destruction of ROS and the control of the oxidative stress, evidencing the higher toxicity that a chronic exposure to CBZ induces in comparison with an acute exposure, especially concerning GLYC and LPO levels and CYP3A4 activity (Almeida et al., 2014).

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