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Ecotoxicological effects of losartan on the brown mussel *Perna perna* and its occurrence in seawater from Santos Bay (Brazil)

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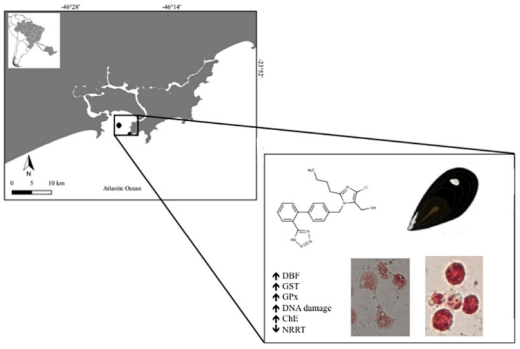
HIGHLIGHTS

- Losartan concentrations in seawater from Santos bay ranged from 0.2 to 8.6 ng/L.
- Reproductive parameters were altered after acute exposure up to 75 mg/L.
- Cyto-genotoxic effects observed after short-term exposure (48–96 h) to ng/L
- *Perna perna* is a sensitive model for assessing losartan toxicity.
- Lysosomal membrane stability was the most sensitive endpoint.

GRAPHICAL ABSTRACT



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ABSTRACT

The antihypertensive losartan (LOS) has been detected in wastewater and environmental matrices, however further studies focused on assessing the ecotoxicological effects on aquatic ecosystems are necessary. Considering the intensive use of this pharmaceutical and its discharges into coastal zones, our study aimed to determine the environmental concentrations of LOS in seawater, as well as to assess the biological effects of LOS on the marine bivalve *Perna perna*. For this purpose, fertilization rate and embryolarval development were evaluated through standardized assays. Phase I (ethoxyresorufin O deethylase EROD and dibenzylfluorescein dealkylase DBF) and II (glutathione S-transferase GST) enzymes, glutathione peroxidase (GPx), Cholinesterase (ChE), lipoperoxidation (LPO) and DNA damage were used to analyze sublethal responses in gills and digestive gland of adult individuals. Lysosomal membrane stability was also assessed in hemocytes. Our results showed the occurrence of LOS in 100% of the analyzed water samples located in Santos Bay, Sao Paulo, Brazil, in a range of 0.2 ng/L–8.7 ng/L. Effects on reproductive endpoints were observed after short-term exposure to concentrations up to 75 mg/L. Biomarker responses demonstrated the induction of CYP450 like activity and GST in mussel gills exposed to 300 and 3000 ng/L of LOS, respectively. GPx activity was also increased in concentration of exposure to 3000 ng/L of LOS. Cyto-genotoxic effects were found in gills and hemocytes exposed in concentrations up to 300 ng/L. These results highlighted the concern of introducing this class of contaminants into marine

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environments, and pointed out the need to include antihypertensive compounds in environmental monitoring programs.

1. Introduction

The current number of elderly people in the world is estimated to be approximately 901 million (equivalent to 12.3% of the world population) but this number is continuously increasing. The scenario for 2050 is that the population of the elderly surpasses two billion, representing about 22% of the global population (Francisco, 2017). The increasing age of the population, stress, sedentary lifestyle, diet habits are directly associated with high blood pressure (Brazilian Archives of Cardiology, 2016), which makes the use of antihypertensive drugs more frequent. Nowadays it is estimated that over a billion adults worldwide are hypertensive and this figure is projected to reach 1.56 billion by 2025 (Jarari et al., 2016).

In this sense, the angiotensin II receptor antagonist class (ARA) has been widely prescribed (Bayer et al., 2014; Knopf and Grams, 2013). In number of prescriptions in United States of America (USA), antihypertensive drugs were classified as "Top 1" from 2011 to 2015 (IMS, 2016). Gu et al. (2012) performed a compilation of data from The National Health and Nutrition Examination Surveys (NHANES - USA) and verified that between 2001 and 2010 there was a 100% increase in the use of ARA. In this period the pharmaceuticals valsartan and losartan (LOS) were classified as the seventh and ninth most used antihypertensive drugs. LOS has also had a significant increase in the consumption in Brazil (23.34%) since 2010, emerging as the main hypertensive drug freely distributed throughout the public health network (Silva et al., 2017).

Taking into account the wide use of this therapeutic class, it is important to point out the wastewater as a main source of aquatic contamination due to the absence or inefficacy of wastewater treatment plants (WWTPs) (Larsson et al., 2007; Bayer et al., 2014). Gurke et al. (2015) determined for LOS a removal rate within a range of 50% to 80% in a sewage treatment plant (STP) including coarse and fine screens, a grit chamber with integrated fat trap, primary clarifiers, biological nitrogen removal and chemical precipitation of phosphorus. The occurrence of Losartan (LOS), an antihypertensive of the ARA class, has been detected in effluents of WWTPs, water supply and environmental matrices (Huerta-Fontela et al., 2011; Godoy et al., 2015a). In samples of a municipal effluent in India, which receives wastewater from bulk drug manufactures, LOS was detected in concentrations ranging from 2400 to 2500 μg/L (Larsson et al., 2007). A study carried in Portugal by Santos et al. (2013) revealed the occurrence of LOS in concentration ranging from 59 to 910 ng/L in hospital effluents while the maximum concentration detected in samples of a STP was 364 ng/L. Gros et al. (2017) detected concentration of LOS varying from 705 to 980 ng/L in effluents of a medium scale WWTP in Sweden. In the same study, in a large scale WWTP, concentrations of LOS of 450 ng/L and 270 ng/L in influent and effluent samples were detected, respectively. Gurke et al. (2015) found a maximum concentration of 333 ng/L in municipal effluent samples in Germany. LOS was also detected in supply water (Spain) with a maximum concentration of 620 ng/L (Huerta-Fontela et al., 2011). In a Brazilian coastal region (São Paulo), Pereira et al. (2016) found LOS in concentrations ranging from 11.8 ng/L to 32 ng/L.

With regard to ecotoxicological studies of antihypertensives, Godoy et al. (2015a) pointed out a lack of data related to this therapeutic class, with 60% of the studies conducted until 2014 employing only acute standardized toxicity tests to assess biological effects. The same authors concluded that there was a need of more studies on the potential risk of antihypertensives in marine/estuarine ecosystems.

Filter feeding organisms with sessile habits and wide distribution have been used as sentinel organisms in ecotoxicological studies and marine biomonitoring (Gerges, 1994). In Brazil, the brown mussel *Perna perna* has been widely used both as seafood and as sentinel organisms in monitoring of anthropogenic pollution trends in coastal waters (Cortez et al., 2012; Trevisan et al., 2014; Pereira et al., 2014; Ortega et al., 2018).

Based on previous studies, LOS monitoring in aquatic environments should be considered, taking into account factors such as (i) its occurrence in wastewater and environmental matrices; (ii) it is one of the antihypertensive drugs mostly used in different regions; (iii) increasing density of the elderly population leading to higher environmental concentrations in near future scenarios.

In this scenario of high consumption and previous detection of LOS in São Paulo coastal zone (Pereira et al., 2016), our study measured environmental concentrations of LOS in Santos Bay and employed ecotoxicological assays to elucidate metabolism and biological responses in different life stages of the brown mussel *Perna perna*.

2. Methods

2.1. Chemical

Standard of LOS 2 Butyl 4 chloro 1 {[2′ (1H tetrazol 5 yl) (1,1′ biphenyl) 4 yl]methyl} 1H imidazole 5 methanol monopotassium salt, (CAS number 124750-99-8, purity ≥98%) as well as all other chemicals employed in this study were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Study area and water sampling

The estuarine region of Santos and São Vicente is located in São Paulo coastal zone, southeastern Brazil. It has an industrial complex; the largest port in Latin America and it is a touristic and densely populated area, where domestic sewage is collected, preconditioned and discharged via submarine outfalls 4.5 km away from the beach in Santos Bay (Fig. 1).

Water samples from the water column (surface — S and bottom — B) were collected at each sampling station in March 2017, in the vicinities of the submarine sewage outfall in Santos Bay, considering the possibilities of effluent plume dispersion. The sampling stations were defined according to the study carried out by Pereira et al. (2016), except station 6. At each sampling station, 3 L of S (−1 m) and B water (−8 m) were collected by using a Van Dorn bottle. The samples were placed into amber glass bottles previously cleaned with HNO_3, methanol and distilled water and then transported to the laboratory in an insulated box with ice (<6 °C) and placed in a freezer at −20 °C until processing time.

2.3. Sample preparation and LC–MS/MS analysis

Seawater samples (field) and water samples from bioassays (test solutions) were prepared according to Pereira et al. (2016). Briefly, the pH of each sample was adjusted to 7.0 ± 0.5 prior to extraction, using an HCl solution (1 M) and then samples were filtered through Whatman filter paper (GF/C diameter 47 mm, particle retention 1.2 μm, Merck, Darmstadt, Germany). The filters were washed with 2 mL of methanol and the methanol extract collected was added to the filtered sample. The samples were then submitted to solid phase extraction using Chromabond HR-X cartridges (3 mL, 200 mg, Macherey-Nagel, Düren, Germany). The SPE cartridges were pre-conditioned with 5 mL of methanol and 5 mL of Milli-Q water and the filtered samples (mixed with

Fig. 1. Sampling stations in Santos Bay (São Paulo, Brazil).

methanol extract) were loaded into the cartridges. After the samples were loaded, the cartridges were rinsed with 5 mL of Mili-Q water (2×) and then dried under vacuum for 30 min. The elution step was performed with 5 mL of acetone and twice with 5 mL of methanol. After elution, the samples were dried under nitrogen flow, resuspended in 1 mL with a solution of water/acetonitrile (95:5, v/v) and filtered in a 0.45 μM filter (Millipore) before MS analysis.

2.4. LC–MS/MS analysis

For analyses of field seawater samples, 10 μL of each sample were analyzed by an HPLC Agilent 1260 (Agilent Technologies, CA, USA) combined with a 3200 QTRAP hybrid triple quadrupole/LIT (linear ion trap) mass spectrometer ABSciex, Ontario (Canada). Seawater samples were analyzed by an Agilent Eclipse XDB-C18 4.6 × 50 mm, 1.8 μm column at 25 °C, and the mobile phase was in 0.1% formic acid (Sigma-Aldrich LC–MS Grade) in water (solvent A) and acetonitrile (J.T. Baker LC–MS Grade) (solvent B). A linear gradient of 0.7 mL·min^{-1} was used, starting with a mixture of 95% solvent A and 5% solvent B. The solvent A percentage was decreased linearly from 95% to 5% over the course of 5 min and this condition was maintained for 1 min. The mixture was then returned to the initial conditions over the course of 2 min, as described in Pereira et al. (2016). LOS was detected and quantified using ESI ionization (positive mode) in Multiple Reaction Monitoring (MRM) mode (Table 1), according Pereira et al. (2016). The ion source parameters were (i) curtain gas - 20 a.u; (ii) collision gas - 8 a.u; (iii) ion spray voltage - 5500; (iv) source gas temperature - 650 °C; (v) ion source gas 1–45 a.u; (vi) ion source gas 2–65 a.u. A matrix-matched calibration curve was employed, as described by Wille et al. (2010).

For determining the LOS concentration in the test solutions from bioassays, 1 L of the spiked water was collected at the beginning of the experiment, then the same procedures adopted for the field seawater samples were applied, and mass spectrometry analyses were performed using a Varian 310 Triple-Quadrupole mass spectrometer (Varian Inc., Walnut Creek, CA) with an ESI source (ESI-MS), by direct infusion. Data acquisition was controlled with Varian MS Workstation version 6.9 (Varian Inc.). Sample analysis was carried out in positive ESI mode with a needle voltage of 20 kV. The capillary temperature was 200 °C, the drying gas pressure was 20 psi and the nebulizing gas pressure was 40 psi. LOS was detected and quantified using Multiple Reaction Monitoring (MRM) mode, with the selection of a precursor ion (423.2 *m/z*) and two ion products to quantify and qualify LOS (207.2 and 405.2 *m/z*, respectively). A matrix-matched calibration curve was employed, as described by Wille et al. (2010).

Table 1
Parameters of multiple reactions monitoring for the positive ion mode, limit of detection, limit of quantification and retention time.

Compounds	Q1	Q3	DP (V)	CE (V)	CXP (V)	LOD (ng/L)	LOQ (ng/L)	RT (min)
Losartan	423.2	207.2	21	31	6	0.01	0.04	4.84
		405.2	21	17	4			

Q1 (first quadrupole); Q3 (last quadrupole); DP (declustering potential); CE (collision energy); CXP (collision exit potential); LOD (limits of detection); LOQ (limits of quantification); RT (retention time); MIM (multiple ion monitoring). In Q3, in the upper cell is the quantifier ion and in the lower cell is the qualifier ion.

2.5. Mussel acclimation and maintenance conditions

Adult mussels (average size 6.2 ± 1,3 cm) were purchased from a mussel farming located in Cocanha Beach (Caraguatatuba, SP, Brazil), due to its good environmental quality, where no water or sediment contamination have been reported by the State Environmental Agency (CETESB, 2016). The organisms were acclimatized for one week (300 L tank), receiving food supply (microalgae), and kept in tanks under constant temperature (24 ± 2 °C), aeration and filtration system.

2.6. Toxicity assays

2.6.1. Fertilization assay

Fertilization assay was performed following USEPA (1991) protocol adapted to *Perna perna* according to Zaroni et al. (2005). The gametes (eggs and sperm) were obtained by thermal stimulation (from 10 °C to 30 °C) of 20 individuals during 30 min. As soon as the organisms started releasing the gametes, they were removed from the tray to

prevent fertilization. The gametes from 3 males and 3 females were collected separately and transferred to glass beakers.

A stock solution of 1000 mg/L was prepared in filtered seawater (0,22 μm membrane) and from this solution all the LOS tested concentrations were prepared. The sperm was exposed to concentrations of 31.25; 62.5; 125; 250 and 500 mg/L for 60 min, in quadruplicate. After this period, a suspension containing approximately 2000 ovules was added to the test recipients. Forty minutes after adding the eggs, the assay was finished by adding 0.5 mL of formaldehyde in each replicate. The first 100 eggs from each replicate were analyzed and fertilization was identified by observation of the occurrence of the membrane of fertilization or first cellular divisions. The results were expressed by the concentration that inhibited the fertilization rate in 50% of exposed organisms (IC50; 1 h).

2.6.2. Embryo-larval development assay

In order to assess the embryolarval development rate in mussels zygotes exposed to LOS, experiments were performed according to the protocol recommended by ASTM (1992) for mussels, with minor adaptations proposed by Zaroni et al. (2005) concerning salinity, which was elevated to 35 ± 1 ppm. Twenty adult individuals were induced to spawn by thermal stimulation. The gametes from 3 males and 3 females were collected separately and transferred to glass beakers. The fertilization was obtained by adding 2 mL of sperm solution to the 200 mL of ovules solution. The rate of fertilized eggs was estimated with the support of Sedgwick-Rafter chamber, and about 500 embryos were transferred to glass tubes containing different nominal concentrations of LOS (5; 10; 25; 50; 75 and 100 mg/L), for a period of 48 h at a temperature of 25 °C and salinity of 35 ppm.

After the exposure period, the assay was finished by adding 0.5 mL of formaldehyde, and the first 100 larvae were analyzed for each replicate. Larvae developed to D-phase were considered normal and a mean percentage of normal development was obtained for each tested concentration. Thereafter were calculated: (i) the concentration of LOS that caused inhibition of the development of the embryos to 50% of the exposed organisms (IC50; 48 h); (ii) the highest concentration tested of LOS that did not cause adverse biological effects on exposed organisms - "NOEC" (No Observable Effect Concentration); and (iii) the lowest LOS concentration tested that caused significant adverse biological effects on exposed organisms - "LOEC" (Lowest Observable Effect Concentration).

2.7. Biomarkers assay

2.7.1. Mussel exposure

The mussels were acclimatized for one week to clean seawater under controlled conditions. After this period, the organisms (n = 21) were exposed in aquaria with different concentrations of LOS (30; 300 and 3000 ng/L) and water control, in triplicate, for 96 h. These concentrations were set according to studies that detected LOS in effluents and surface waters.

In each replicate of all treatments, 7 organisms were exposed in each aquarium containing 10 L of the test solution. Since LOS has been considered a stable molecule with low hydrolysis and biodegradation process (FDA, 2002), controls and test solutions were renewed each 48 h. The physico-chemical parameters were controlled during the experiment without significant changes (salinity 35 ppm, dissolved oxygen 8 ± 0.5 mg/L, pH 8.3–8.5). The natural seawater used in the assay was filtered through a membrane of 200 μm in order to filter higher particulates and maintain a food supply (phytoplankton) because during the experiment no other type of food was provided.

2.7.2. Tissue extraction and storage

The hemolymph, gill and digestive gland tissues were extracted for analysis at T0, T48 h and T96 h. Organisms were removed from each replicate of the different treatments, totaling 10 mussels for T48 h and 10 mussels for T96 h. Immediately after removal of the hemolymph, the slides were prepared for analysis of the lysosomal membrane stability (LMS). After this procedure, gills and digestive glands were dissected, separated into microtubes and stocked in ultrafreezer (−80 °C) until the biomarker analyses were carried out.

2.7.3. Neutral red retention time assay (NRRT)

NRRT assay was performed following the method described by Lowe and Pipe (1994) to assess the lysosomal membrane stability (LMS).

The mussels were removed from the aquariums and with the aid of a syringe containing physiological saline solution (pH 7.3) 40 μL of hemolymph from each organism was withdrawn and placed on glass slides, which were transferred to a dark and humid chamber for 15 min to promote cell attachment. Then, the excess liquid was removed and 40 μL of the neutral red dye (NR) were added onto all the slides. After another 15 min of incubation the slides were analyzed periodically (every 15 min). The endpoint was the time when at least 50% of the examined cells by optical microscopy (400×) exhibited dye loss from the lysosomes to the cytosol or structural abnormalities.

2.7.4. Tissue preparation

Gills and digestive glands from each organism were defrosted on ice and homogenized with 4 times the volume of 100 mM NaCl buffer, 25 mM HEPES-NaOH, 0.1 mM EDTA, 0.1 mM DTT, pH 7.5, in a homogenizer (Tissue Tearor). After homogenization, the extract was centrifuged at 4 °C at 15,000*g* for 20 min, thus obtaining the supernatant fraction (15,000*g*) where the activities of CYP450 like (Ethoxyresorufin O deethylase - EROD and Dibenzylfluorescein dealkylase - DBF), Glutathione-S-transferase (GST), Glutathione peroxidase (GPX), Cholinesterase (ChE) were analyzed, as well as the concentration of proteins in the cytoplasmic cell fraction, according to the method of Bradford (1976). An aliquot of homogenized tissue was separated for lipoperoxidation (LPO) and DNA damage analyzes, and the protein concentration in this aliquot was also evaluated by the method of Bradford (1976).

2.7.5. Ethoxyresorufin O deethylase (EROD)

The EROD activity was evaluated by the adapted test of Gagné and Blaise (1993). The transformation of 7 hydroxyresorufin in resorufin (EROD activity) was determined fluorometrically using 520 nm (excitation) and 590 nm (emission) filters. The determination of 7 hydroxyresorufin in the samples was performed using a standard calibration curve of 7 hydroxyresorufin. The results were expressed as pmol/min/mg protein.

2.7.6. Dibenzylfluorescein dealkylase (DBF)

The determination of DBF activity was performed according the method described by Gagné et al. (2007) using as substrate 10 μM dibenzylfluorescein and incubated with a solution of 1 mM NADPH in a test solution (50 mM NaCl containing 10 mM HEPES-NaOH, pH 7.4). The fluorescence of the sample was measured with a 485 nm excitation and 516 nm emission filter. Results were expressed in pmol/min/mg protein.

2.7.7. Glutathione S-transferase activity (GST)

The method used to determine GST activity was adapted from Mc Farland et al. (1999). The activity was analyzed using 42 mM 1 chloro 2.4 dinitrobenzene (CDNB), 1 mM GSH as substrate and measured at 340 nm every 30 s for 3 min. Results were expressed as OD/min/mg proteins.

2.7.8. Glutathione peroxidase activity (GPx)

The methodology used to determine GPx activity was developed according to the method proposed by Mc Farland et al. (1999). GPx activity was measured at 340 nm every 2 min for 10 min, using 1 mM cumene hydroperoxide as the substrate. The decrease in absorbance of

NADPH measured at 340 nm during the oxidation of NADPH to NADP+ was indicative of GPx activity. Results were expressed as nmol/min/mg protein.

2.7.9. DNA damage

DNA damage was evaluated by the Olive (1988) alkaline precipitation assay, using fluorescence to quantify traces of DNA (Gagné and Blaise, 1993). Fluorescence was measured using 360 nm filter (excitation) and 450 nm (emission) and a salmon sperm genomic DNA standard (Sigma) was employed for calibration. The results were expressed in μg/mg protein.

2.7.10. Lipid peroxidation

Analysis of lipid peroxidation was performed by the thiobarbituric acid method (Wills, 1987). This determination was employed by fluorescence using 516 nm (excitation) and 600 nm (emission). The tetramethoxypropane standards were prepared in homogenization solution. The results were expressed in μM TBARs/mg proteins.

2.7.11. Cholinesterase (ChE)

The analysis of the ChE activities of the gills and digestive glands were performed according to the method described by Ellman et al. (1961) using a concentration of 0.3 mM acetylcholine iodide in the enzyme assay. The variation of absorbance per minute at 412 nm at 25 °C was recorded in a spectrophotometer. The results were expressed in μmol DNTB/min/mg protein.

2.8. Statistical analysis

For the fertilization assay, an EC_{50} was calculated by Trimmed Spearman-Karber. The linear interpolation method was used to calculate the IC_{50} (48 h) for the embryo-larval development assay. *t*-Test was employed to assess differences between T0 and water controls. Since no difference was detected, water controls were used as references for 48 h and 96 h. *One-way* ANOVA followed by the Dunnett's test was used to identify the concentrations significantly different of water controls. Statistical differences were considered significant when $p \leq 0.05$. The software Prism v.7 was employed for ANOVA and *pos hoc* analysis.

3. Results

3.1. Environmental concentrations

Table 2 shows the environmental concentrations of LOS. This pharmaceutical compound was detected in all sampling stations including the reference area.

3.2. Fertilization rate and embryo-larval development assays

The measured concentrations of LOS at the beginning of the exposure experiment for the fertilization and embryo-larval assays (T0) are shown in Table 3.

The EC50 1 h for the rate of fertilization of *P. perna* was calculated as 219.2 mg/L, with a confidence interval ranging from 208.3 to 231.8 mg/L. The normal embryo-larval development of 50% exposed zygotes was inhibited in the concentration of 84.6 mg/L (IC50 48 h) with a confidence interval ranging from 62.8 to 87.5 mg/L, while NOEC and LOEC were 50 mg/L and 75 mg/L, respectively.

Table 2
Environmental concentration of LOS in surface and bottom water samples (1–6 sampling stations) from Santos Bay.

	LOS concentration (ng/L)					
	1	2	3	4	5	6
Surface	8.70	3.89	2.10	0.295	3.62	0.60
Bottom	2.46	1.59	1.50	1.18	1.07	1.79

Table 3
Nominal and measured concentrations of LOS in fertilization and embryo-larval assays (T0).

Nominal concentration (mg/L)	Measured concentration (mg/L)
0	<LOD
5.0	4.72
25.0	23.43
75.0	71.13
125.0	118.36
250.0	232.92
500.0	469.36

LOD - limits of detection.

3.3. Biomarkers responses

The nominal and the measured concentrations of LOS at time T = 0 are reported in Table 4. The nominal concentrations were similar to those determined at T = 0.

The lysosomal membrane stability (LMS) showed a concentration-time response, with a significant decrease in the NR retention time after exposure to 3000 ng/L in 48 h, and down to 300 ng/L after 96 h (Fig. 2).

The activities of EROD, DBF, GST, GPX and ChE as well as the DNA damage and LPO in the gill tissue are shown in Fig. 3.

When gills were evaluated, the activity of EROD and LPO did not show significant difference in relation to the control in any of the analyzed times. With regard to the activity of the DBF, only the concentration of 300 ng/L showed significant difference, with induction of the activity after 96 h. Significant increase of GST, GPx activities, as well as DNA primary damages were found after 48 h exposure to 3000 ng/L. With regard to the ChE, an induction of the activity of this enzyme in the concentration of 3000 ng/L at 96 h of exposure was observed.

The activities of EROD, DBF, GST, GPX and ChE as well as the DNA damage and LPO in the digestive glands are shown in Fig. 4. Significant differences were only detected to EROD (inhibition of activity) in mussels exposed to 300 ng/L and 3000 ng/L after 96 h.

4. Discussion

The pharmaceuticals represent a major group of emerging pollutants, which have been found in freshwater and marine environments (UNESCO, 2017), representing a global challenge to water quality in terms of environmental status and human supply. Their occurrence in the aquatic compartment leads to the need of knowledge about possible harmful effects of this class of substances on the biota.

LOS was quantified in different rivers of the Iberian Peninsula in a concentration ranging from 0.17 ng/L to 220.63 ng/L (Osorio et al., 2016). In the marine environment, Moreno-González et al. (2015) detected concentrations of 104 ng/L and 6.47 ng/g in samples of water and sediment from the Spanish coast, respectively. In a tropical coastal zone (Santos Bay, Brazil) LOS concentrations varied from 11.8 ng/L to 32 ng/L in marine water (Pereira et al., 2016). In the present study, the concentrations of LOS detected in surface and bottom samples ranged from 0.29 ng/L to 8.70 ng/L. These concentrations were lower than those reported in the study performed by Pereira et al. (2016);

Table 4
Nominal and measured concentrations of LOS (T0) in the experiments with biomarkers.

Nominal concentration (ng/L)	Measured concentration (ng/L)
30	27.3
300	276.9
3000	2811.2

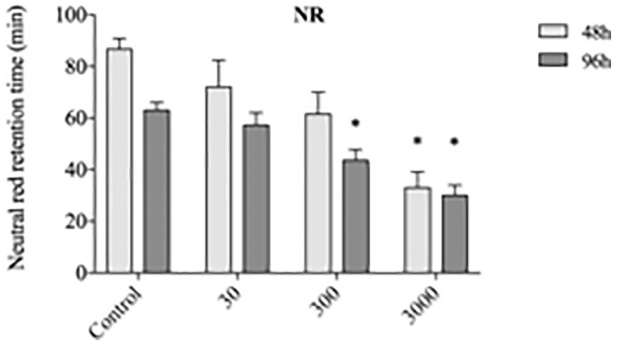

Fig. 2. Neutral red retention time assay (mean ± SE). An asterisk indicates a significant difference from the control (ANOVA - Dunnett's, $p < 0.05$).

however, these authors detected LOS only in 30% of samples collected three years before, whereas the present study has found LOS in 100% of samples, including the farthest station of the effluent discharged (station 6). Gros et al. (2012) also detected LOS in marine surface water (Spain) in a similar range found in the present study. The frequent occurrence of this antihypertensive in Santos bay could be related to aging population, which according Bersusa et al. (2010), 79.3% of the hypertensive people in Santos are over 40 years old, and according to the Brazilian Institute of Geography and Statistics (IBGE, 2010), the cities of Santos and São Vicente make up 82.3% of the population at this age group.

With regard to adverse biological effects, we have employed standardized ecotoxicology assays and a suite of biomarkers responses of a marine invertebrate. *P. perna* mussel is considered a key species in Brazilian rocky shores and broadly cultivated for human consumption. The results obtained in the fertilization and embryo-larval assays fit

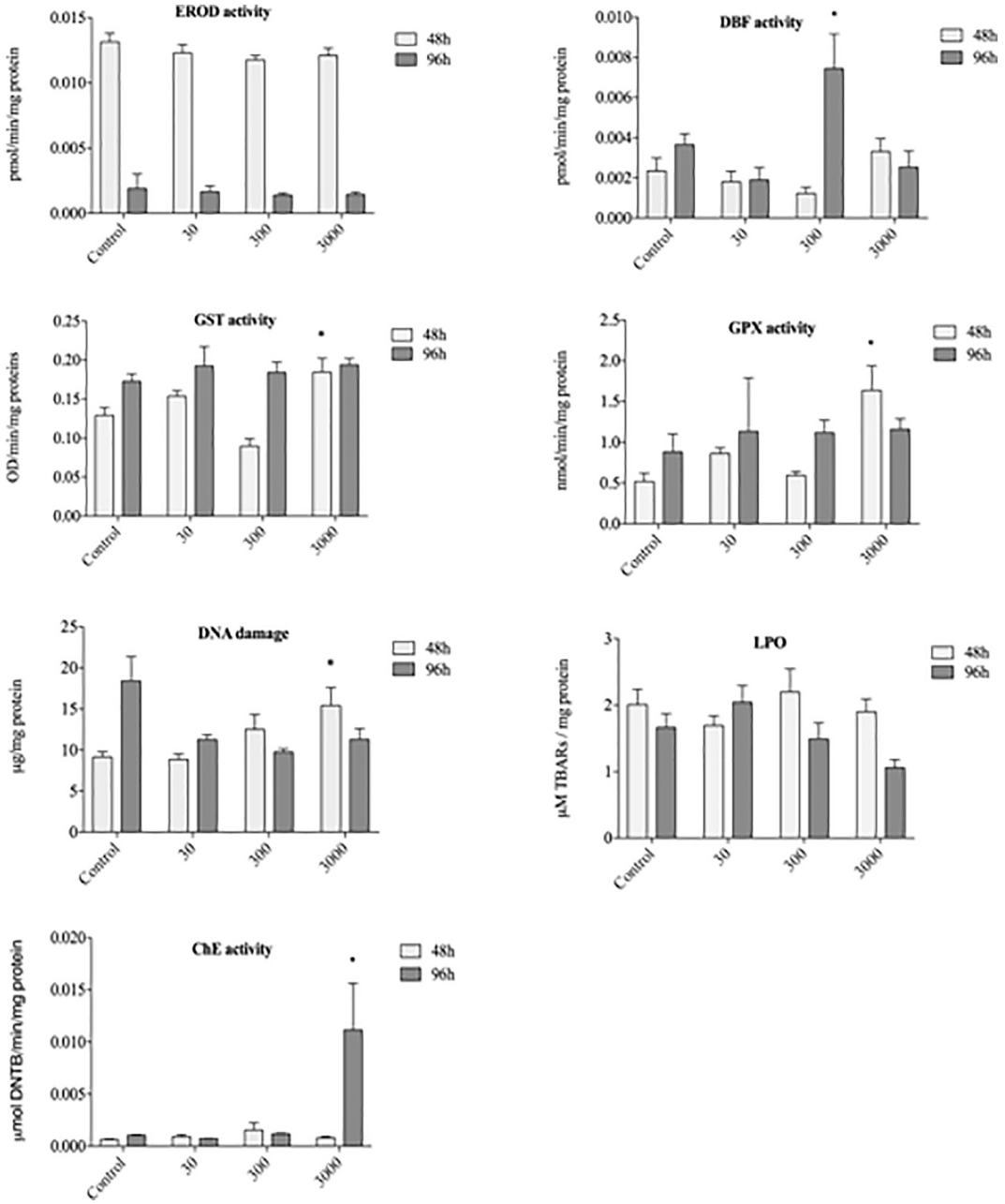

Fig. 3. Biomarker responses in gill tissues (mean ± SE). An asterisk indicates a significant difference from the control (ANOVA - Dunnett's, $p < 0.05$).

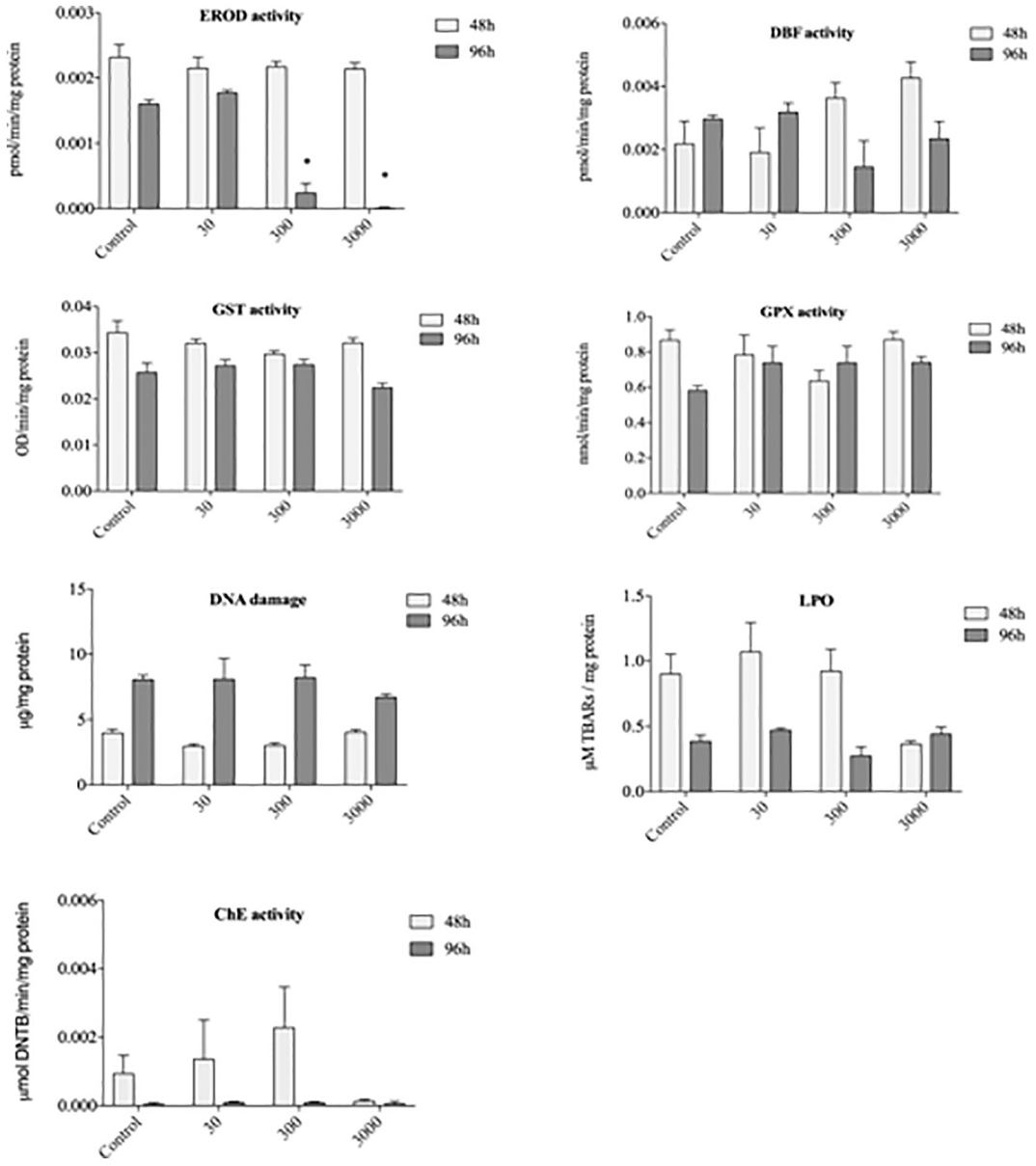

Fig. 4. Biomarker responses in digestive gland (mean ± SE). An asterisk indicates a significant difference from the control (ANOVA - Dunnett's, $p < 0.05$).

Table 5
Toxicity assays with LOS and aquatic organisms of different trophic levels.

Losartan					
Species	*Endpoint*	LC50/EC50 mg/L	NOEC mg/L	LOEC mg/L	References
Daphnia sp.	Lethal effects	331	80	nd	FDA (2002)
Pimephales promelas		>1000	100	nd	
Oncorhynchus mykis		>929	>929	nd	
S. capricornutum	Cell growth	nd	143	nd	
Lemna minor	Growth rate	No data	0.78	1.56	Godoy et al. (2015b)
	Frond number	63.7	nd	nd	
	Total frond area	64.6	nd	nd	
	Fresh weight	76.9	nd	nd	
Lytechinus variegatus	Embryo-larval development	nd	50	70	Yamamoto et al. (2014)

nd - no data.

into the same order of magnitude (mg/L) in relation to the previous studies (Table 5).

The bivalve *Perna perna* presented values of NOEC (50 mg/L) and LOEC (75 mg/L) similar to those found in a study employing the sea urchin *Lytechinus variegatus* (Yamamoto et al., 2014). Considering all the previous studies, it is possible to note that the aquatic macrophyte *Lemna minor* shows a relatively higher sensitivity to LOS. Another antihypertensive pharmaceutical of the "sartans" group (Valsartan) has been found in the aquatic environment (Klosterhaus et al., 2013; Bayer et al., 2014). Bayer et al. (2014) found EC50 (72 h) of >115 mg/L and NOEC of 85 mg/L to microalgae *Desmodesmus subspicatus* exposed to Valsartan. In the same study effect concentrations for the fish *Oncorhynchus mykiss* and for *Daphnia magna* of >100 mg/L and >580 mg/L, respectively, were reported. Using the sea urchin *Lytechinus variegatus*, Yamamoto et al. (2014) conducted a study with Valsartan and observed NOEC of 12.5 mg/L and LOEC of 25 mg/L. This author related the higher Log K_{ow} of Valsartan (3.65) to its higher toxicity when compared to LOS (Log K_{ow} 1.19), considering that such property determines a higher capacity to bioaccumulate and reach target organs.

The data obtained through the chemical analysis to determine the real concentrations of LOS in the biomarkers assays were similar to the nominal concentrations. Thus, the nominal concentrations were employed to calculate NOEC and LOEC.

Biomarker responses shed light on the metabolism and sub-lethal effects of the antihypertensive LOS in a non-target marine organism. The activity of DBF was induced in the gills after 96 h of exposure at 300 ng/L, whereas GST was induced in gills of mussels exposed to 3000 ng/L after 48 h. The metabolism of this pharmaceutical in vertebrates occurs through the Cytochrome P450 (CYP 450) system specifically through the CYP2C9 and CYP3A4 families (Ripley and Hirsch, 2010), which is in agreement with our results obtained in a marine bivalve. Phases I and II of the detoxification system produce reactive oxygen species (ROS) and metabolites, which are able to promote oxidative stress and cellular damages. This fact can induce the activity of antioxidant enzymes to protect against oxidant damages (Maranho et al., 2014).

The activity of GPx was also induced at 3000 ng/L (48 h), which shows a correlation with ROS generated by phase I detoxification system. The data on sub-lethal effects, especially DNA damage and ChE activity, suggest a possible overlapping of the antioxidant system, since these effects were estimated in the same concentrations (3000 ng/L). The enzymatic activity of the ChE is considered a biomarker of neurotoxicity effects and, in bivalve mollusks the alterations in the activity of this enzyme can be related to the control of the closure of the valves, alterations in the muscle movements, in the ciliary beating, among others (Viarengo et al., 2007). Previous studies with pharmaceuticals reported the induction of the activity of ChE in bivalve mollusks (Mesquita et al., 2011; Gonzalez-Rey and Bebianno, 2014) a fact that coincides with the data obtained for LOS in the present study. Zhang et al. (2002) reported that the induction of ChE is associated with cellular apoptosis in several human cells and other mammals, possibly because ChE is released after the rupture of the cellular membrane. It is possible that the induction of the activity of ChE is related to the membranothropic effects and the resulting cellular apoptosis caused by the LOS. The reduced stability of the lysosomal membranes confirms this hypothesis, since dependent concentration-time responses were found after exposures up to 300 ng/L.

Furthermore, toxicological studies reported the ability of the LOS to inhibit H^+ ATPase activity in rodent kidney cells (Valles and Manucha, 2000) and the capacity of this pharmaceutical to interact with the bilayer cell membrane altering the fluidity of membrane (Zoumpoulakis et al., 2003). These characteristics may also be related to effects detected on the lysosomal membrane stability. This biomarker showed the most sensitive response, which coincides with previous studies on the cytotoxicity of pharmaceuticals compounds (Cortez et al., 2012; Pusceddu et al., 2018). Aguirre-Martínez et al. (2013) evaluating the toxicity of ibuprofen, carbamazepine and novobiocin on the crab *Carcinus maenas*, concluded that stability of lysosomal membrane is a good indicator of general stress in organisms exposed to pharmaceuticals at environmentally relevant concentrations.

With regard to the sensitivity of the different mussel's life stages used in the present study, the endpoints fertilization rate and embryolarval development, although considered phases highly sensitive to environmental pollutants (Beiras et al., 2003), were not responsive to detect adverse biological effects in environmentally relevant concentrations of LOS ranging from ng/L to μg/L. On the other hand, the data obtained with the use of biomarkers in adult mussels showed some significant biological effects (DNA damage and reduced lysosomal membrane stability) after short-term exposures at concentration closer to those found in WWTPs effluents and marine surface water (Larsson et al., 2007; Moreno-González et al., 2015; Pereira et al., 2016; Gros et al., 2017).

Perna perna mussels showed to be a suitable marine model, which could be employed in future environmental assessments. The gill was the most responsive tissue, showing detoxification (DBF and GST activities) and antioxidante defenses (GPx), but this was not able to prevent mitochondrial DNA damage.

5. Conclusion

The pharmaceutical LOS was quantified in 100% of surface and bottom water samples from Santos Bay ranging from 0.295 to 8.70 ng/L. Adverse effects of the antihypertensive Losartan on reproductive parameters of the brown mussel *Perna perna* were detected in higher concentrations (mg/L) after short-term exposure. In spite of the high ecological relevance of these endpoints, they are not expected in realistic scenarios of aquatic ecosystems. However, detoxification and antioxidant systems were induced after exposure to concentrations ranging from ng/L to μg/L, as such as cyto-genotoxic effects in gills and hemolymph. These results highlighted the concern of introducing this class of contaminant into marine environments, and pointed out the need to include antihypertensive compounds as targets to wastewater treatments plants, such as including them in environmental monitoring programs.

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