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Environmental risk assessment of triclosan and ibuprofen in marine sediments using individual and sub-individual endpoints☆

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ABSTRACT

The guidelines for the Environmental Risk Assessment (ERA) of pharmaceuticals and personal care products (PPCP) recommend the use of standard ecotoxicity assays and the assessment of endpoints at the individual level to evaluate potential effects of PPCP on biota. However, effects at the sub-individual level can also affect the ecological fitness of marine organisms chronically exposed to PPCP. The aim of the current study was to evaluate the environmental risk of two PPCP in marine sediments: triclosan (TCS) and ibuprofen (IBU), using sub-individual and developmental endpoints. The environmental levels of TCS and IBU were quantified in marine sediments from the vicinities of the Santos submarine sewage outfall (Santos Bay, São Paulo, Brazil) at 15.14 and 49.0 ng g^{-1}, respectively. A battery (n = 3) of chronic bioassays (embryo-larval development) with a sea urchin (*Lytechinus variegatus*) and a bivalve (*Perna perna*) were performed using two exposure conditions: sediment-water interface and elutriates. Moreover, physiological stress through the Neutral Red Retention Time Assay (NRRT) was assessed in the estuarine bivalve *Mytella charruana* exposed to TCS and IBU spiked sediments. These compounds affected the development of *L. variegatus* and *P. perna* (75 ng g^{-1} for TCS and 15 ng g^{-1} for IBU), and caused a significant decrease in *M. charruana* lysosomal membrane stability at environmentally relevant concentrations (0.08 ng g^{-1} for TCS and 0.15 ng g^{-1} for IBU). Chemical and ecotoxicological data were integrated and the risk quotient estimated for TCS and IBU were higher than 1.0, indicating a high environmental risk of these compounds in sediments. These are the first data of sediment risk assessment of pharmaceuticals and personal care products of Latin America. In addition, the results suggest that the ERA based only on individual-level and standard toxicity tests may overlook other biological effects that can affect the health of marine organisms exposed to PPCP.

1. Introduction

Until recently pharmaceuticals and personal care products (PPCP) were not included in environmental monitoring programs mainly because of their low environmental concentrations and the absence of analytical methodologies to detect them. The concern about the environmental contamination by PPCP began to be part of the agenda of governments after the publication of studies showing fish feminization due to exposure to estrogenic substances (Harries et al., 1997; Jobling et al., 1998; Hinck et al., 2009) and the massive death of vultures caused by the ingestion of diclofenac (Green et al., 2004). These studies were important to trigger concern on the environmental risks of PPCP, which include antimicrobial, anti-inflammatory, contraceptives drugs, antidepressants and antiepileptic (USEPA, 2015). The preoccupation has involved especially - although not exclusively - the aquatic biota, since the water bodies are the final destination of many of these substances (Arnold et al., 2014).

The knowledge about the effects of PPCP on freshwater organisms has evolved significantly (e.g. Fent et al., 2006; Arnold et al., 2014), but there are few empirical data about the ecotoxicity of such compounds to marine organisms nowadays (Gaw et al., 2014).

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This information gap is especially important regarding contaminated sediments and marine or estuarine benthic biota (Brausch and Rand, 2011). Previous studies focusing on marine sediments showed that carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol and propranolol inhibit *Vibrio fischeri* bioluminescence at concentrations ranging from 36.1 to 163.9 ng g^{-1}, and affect the embryo-larval development of sea urchin and the growth rate of marine algae (Maranho et al., 2014, 2015a). Maranho et al. (2015b) also observed lethal and sublethal effects (alterations in cellular energy status, metabolism of monoamines, and inflammation properties) in polychaetes exposed to environmental concentrations of human pharmaceuticals in marine spiked sediments.

Currently, triclosan (TCS) and ibuprofen (IBU) belong to classes of emerging compounds of greatest concern to environmental protection agencies, such as the USEPA (2015) and Environment Canada (2011). Triclosan and ibuprofen have been commonly found in environmental matrices such as surface waters and sediments in concentrations ranging from pg L^{-1} to μg L^{-1} and pg g^{-1} to μg g^{-1}, respectively (Kolpin et al., 2002; Lindström et al., 2002; Agüera et al., 2003; Weigel et al., 2004; Xie et al., 2008; Zhao et al., 2010; Pintado-Herrera et al., 2013).

Given the considerable lack of information about the effects of human PPCP in sediments on marine organisms and considering that standard toxicity tests may not be sensitive enough to see the effects of human PPCP in aquatic biota (Aguirre-Martínez et al., 2015), it is important not only to increase the availability of ecotoxicological data but also to adopt new approaches to assess the environmental risks associated with PPCP in coastal areas (Fabbri and Franzellitti, 2016).

The procedure to conduct an environmental risk assessment (ERA) of PPCP within the regulatory scope by the European Medicine Agency (EMEA, 2006) is based on different types of evaluation. The first level of evaluation demands (i) the estimate of the biota exposure to the studied substance, either by direct measurements from environmental samples (Measured Environmental Concentrations - MEC), or indirectly through a prediction of its environmental concentrations (Predicted Environmental Concentrations - PEC). If environmental risks are expected, then the ERA framework leads to the second level of evaluation: (ii) identification of the final destination of the substance (based on its physical-chemical characteristics) and its ecotoxicological effects. From these data, the Predicted No Effect Concentrations (PNEC) can be estimated and at last (iii) the risk quotient (RQ) is calculated from the ratio between PEC (or MEC) and PNEC. If RQ < 1, further evaluations are not required; if RQ > 1, more refined evaluations are needed, including the evaluation of more sensitive endpoints and the performance of sediment bioassays.

Consequently, the evaluation of the potential effects of PPCP to aquatic organisms plays an important role in the ERA. The use of standardized ecotoxicological assays (e.g. OECD Guidelines for Testing Chemicals) is the most common ecotoxicological approach employed in the scope of the ERA in relation to PPCP (Hernando et al., 2006). Although such assays bring relevant information, they are unable to show a more realistic view of environmental risks of PPCP since the biological responses are quantified only at the individual level. The inclusion of chronic or sub-chronic endpoints, as well as sensitive responses at sub-individual levels are important to evaluate the risks of PPCP, since the most common environmental scenario is a continuous exposure to low concentrations in the marine environment. In addition, the evaluation of effects at lower levels of biological organization (i.e. sub-individual) can predict effects at higher levels (e.g. mortality, population decline, community structure) and may generate information about the mechanisms of action of PPCP in non-target organisms (Villalaín et al., 2001; Martin-Diaz et al., 2009; Pereira et al., 2014).

The current study evaluated the environmental risk of two widely used pharmaceutical substances (TCS and IBU) in three marine invertebrates used in sediment assessments (the mussel *Perna perna* and the sea urchin *Lytechinus variegatus*) including a new alternative sediment sentinel species, the mussel *Mytella charruana*. The biota exposure was firstly estimated by measuring environmental concentration of these substances in sediments from the vicinity of the sewage outfall of Santos Bay, Southeast Brazil. Then the concentration effect of TCS and IBU was established based on chronic and sub-individual endpoints measured in the organisms exposed to spiked sediments. At last, following the environmental risk assessment of EMEA (2006), it was estimated the RQ for each of the substances studied. This study is the first environmental risk assessment for PPCP in marine sediments from an area of the Latin America coast and will contribute to the knowledge of environmental risks associated with these substances in marine tropical ecosystems.

2. Materials and methods

2.1. Chemicals

The bactericidal triclosan (CAS number: 3380-34-5) has a molecular weight of 289.5 g mol^{-1}, water solubility of 10 mg L^{-1}, pKa value of 7.9 and log Kow 4.76 with half live of 40 days (Huang et al., 2015; TOXNET, 2016). The anti-inflammatory ibuprofen (CAS number: 15687-27-1) has a molecular weight of 206.28 g mol^{-1}, water solubility of 21 mg L^{-1}, pKa value of 4.91 and log Kow 3.97 with half live of 19 days (Conkle et al., 2012; TOXNET, 2016). All reagents used in the current study were purchased from Sigma-Aldrich® (purity was ≥98%).

2.2. Exposure assessment

The exposure assessment of TCS and IBU was performed by quantifying the concentration of these compounds in sediments (i.e. MEC estimative) from the vicinity of the sewage outfall of Santos Bay at the coast of São Paulo, Southeastern Brazil (Fig. 1).

2.2.1. Study area and sediment sampling

The Santos and São Vicente estuarine system is located in the coast of the state of São Paulo, Southeastern Brazil. This area comprises a dense urbanization area, the biggest industrial complex located in the Brazilian coastal zone, and also the major Latin American port.

Five sampling sites within the surroundings of the sewage outfall of Santos Bay were established for sediment collection (Fig. 1) to determine the environmental levels of TCS and IBU. These sites were established based on the same criteria adopted by Environmental Agency of São Paulo (CETESB, 2007) to monitor the quality of surface water from the vicinity of the sewage outfall of Santos Bay. The sediment used in the ecotoxicological assays was collected in a reference area (*Toque Toque Grande* beach, in the coast of *São Sebastião*, São Paulo, SE Brazil), located approximately 80 km away from relevant contamination sources (Fig. 1). The Environmental Agency of São Paulo monitored this area weekly in 2014, indicating optimal quality throughout the year (CETESB, 2014).

The sediments were collected in February 2015 using a *van Veen* grab sampler and maintained in plastic bags in a cooler box with ice during the transport to the laboratory, and then kept in the dark at the temperature of 4 °C.

2.2.2. Physical-chemical characterization

The sediments, both from the reference area and the surroundings of the submarine sewage outfall, were analyzed for

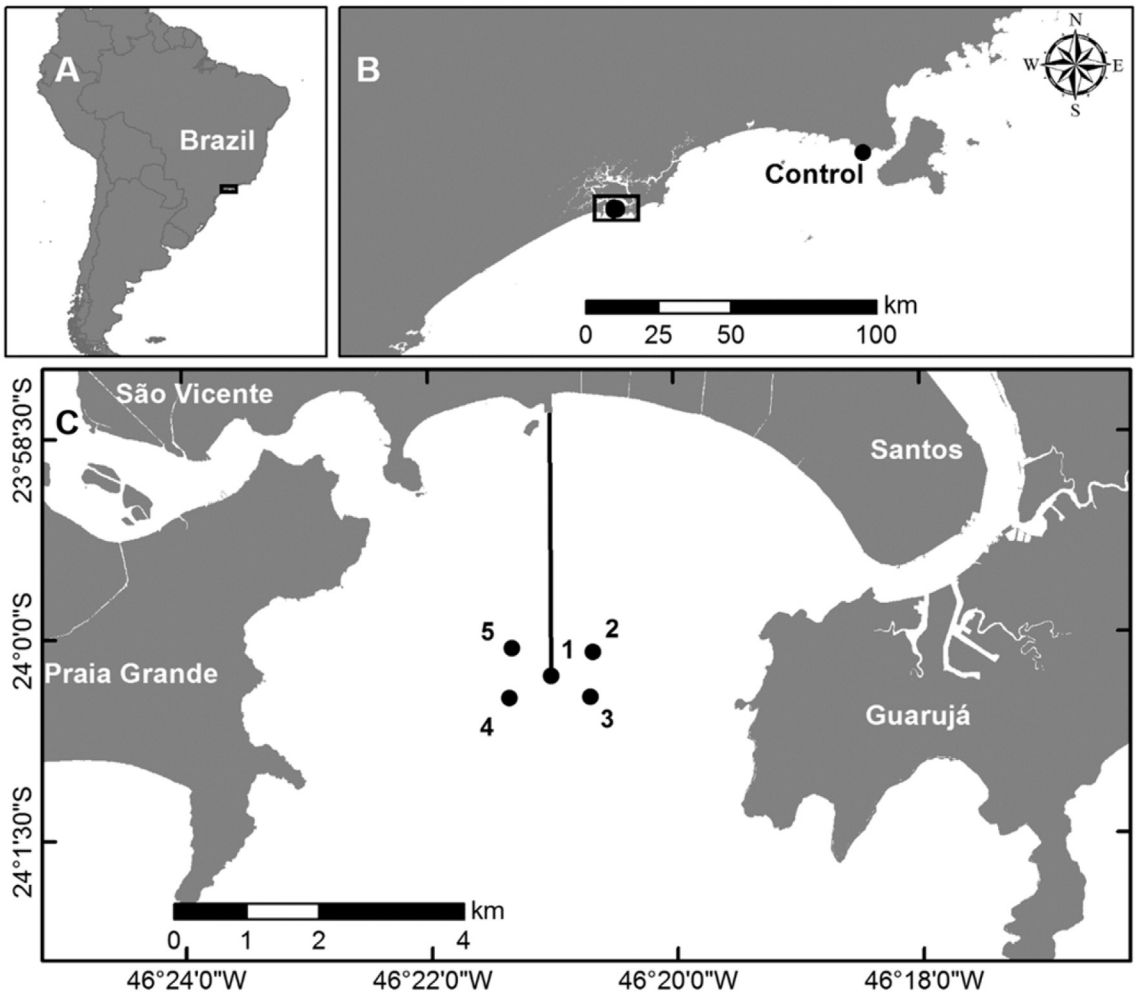

Fig. 1. Sediment sampling area: A) Position in South America; B) Reference site at *Toque Toque Grande* beach, coast of *São Sebastião*, São Paulo, SE Brazil; C) Santos Bay, Santos/SP, SE Brazil.

sediment grain size by dry sieving (Wentworth, 1922), levels of organic matter (OM) by the loss-on-ignition method (Luczak et al., 1997), and levels of carbonates (Hirota and Szyper, 1976).

2.2.3. Sediment spiking and test system preparation

For the spiking procedure, sediment samples were dried at 70 °C to reduce possible interferences of volatile organic compounds (Atkinson and Arey, 2003). The same volume of water lost in this procedure was added to the sediment sample before the spiking procedure. The sediment spiking process was performed following USEPA (2001). Fifty mL of a previously prepared solution of the tested compound with a known concentration was added to 500 g of wet sediment placed in a glass flask. The sediment was kept under agitation for 30 min in a jar-rolling apparatus. The flasks were then maintained for seven days in the dark and at a temperature of 4 ± 2 °C to establish a chemical equilibrium between the tested substance and the sediment (Francis et al., 1984; Löffler et al., 2005).

2.2.4. Chemical analysis

A sample was composed of sediments collected in five sampling sites around the sewage outfall of Santos Bay. The concentrations of TCS and IBU were analyzed by liquid chromatography/electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) on a negative acquisition mode. The concentrations of the studied compounds were also measured in the spiked sediments used in the ecotoxicological assays.

The solid-phase extraction (SPE) method was performed using Xtrata – X 33μ cartridge Polymeric Reversed-Phase (200 mg/3 mL, Phenomenex). Columns were pre-conditioned with 2 mL of acetonitrile; 2 mL of methanol: isopropanol: acetonitrile (1: 1: 1); 2 mL of Milli-Q® water. Twenty five mL of the extract and 250 mL of Milli-Q® water were used for the percolation step of samples in the cartridges. The cartridges were then dried under a vacuum for 5 min. Elution was performed using 3 mL of methanol and 3 mL of acetone: methanol (1:1). Thereafter the eluate was taken to complete dryness under a gentle stream of compressed air to remove the organic solvent from the SPE step. It was then completed with 500 μL of methanol: water (7:3).

After the SPE procedure, the samples were then analyzed in HPLC-MS/MS system in MRM mode: Agilent Eclipse XDB-C18 column (75 mm × 3 mm x 3.5 μm); gradient elution mobile phase was a 5 mM ammonium acetate buffer (solvent A) and methanol (solvent B); temperature = 25 °C; injection volume of 10 μL; flow: 0.4 mL min^{-1}.

A matrix-matched calibration curve was employed and the linearity and accuracy of the analytical method were evaluated using blank sediment samples (not contaminated and prior to the extraction), spiked with seven concentration levels of TCS and IBU (5, 10, 15, 20, 25, 30, 40 ng g^{-1}).

2.3. Effects evaluation

The chronic effects of TCS and IBU were evaluated at the individual level through the embryo-larval development bioassay for two marine species: a bivalve mussel (*P. perna*) and a sea urchin (*L. variegatus*). The embryos of *P. perna* and *L. variegatus* were exposed to TCS or IBU in sediments using two different exposure approaches: (i) the Sediment-Water Interface (SWI), which evaluates the toxicity due to solubilization of the tested substance and the upflow of contaminated pore water and (ii) the Sediment Elutriate (SE), which emulates the sediment resuspension and the possible solubilization of the tested substances. This approach (SWI and SE) represents two real possibilities of occurrence in the study area. The sediment-water interface provided more realistic exposure conditions for epibenthic embryo-larval test organisms (Cesar et al., 2004). On the other hand, the sediments at the Santos Bay are prone to resuspension not only due to natural factors (e.g. tidal currents, storms), but also due to intense port activities, such as the turbulence caused by vessels' traffic and dredging activities.

The dilution water used in the experiments was prepared through the solubilization of marine salt (*Redsea*®) in distilled water to 30 or 35 (depending on the test organism). The salinized water was filtered through a 0.22 μm cellulose membrane.

The SWI test system was set by placing 2 mL of spiked sediment to 8 mL of dilution water in test tubes. A plankton net (0.45 μm) was placed near the sediment to prevent the contact of the organisms with the sediments and to facilitate the recovery of the larvae at the end of the experiment. This test-system was set up 12 h prior to the beginning of the experiments to let the sediment-water interface reach equilibrium.

The SE was obtained through a mixture of spiked sediment and dilution water at a 1:4 ratio (sediment-water). This mixture was vigorously shaken for 30 min and left to rest for 1 h before the beginning of the assays. The supernatant was then carefully withdrawn and placed in test tubes to perform the assays.

For the biological assessment at the sub-individual level, the stability of the lysosomal membrane (through the Neutral Red Retention Time bioassay) was evaluated using adult specimens of the marine mussel *M. charruana* exposed to whole sediments (WS) spiked with TCS or IBU. The whole sediment exposure is able to show the effects of contaminants is organisms in direct contact with the sediment matrix. The exposure of *M. charruana* to the WS was performed by placing 300 g of the spiked sediment with different concentrations of the test substance in glass jars and then 1.2 L of dilution water was carefully added to set up the test system.

A negative sediment control was run for each assay. Each treatment had 4 replicates and a battery of three trials was performed for each assay.

2.3.1. Perna perna toxicity assays

The toxicity assay to evaluate chronic effects to the embryo-larval development of *P. perna* was performed according to Zaroni et al. (2005). The method consisted of inducing the release of gametes by means of water temperature variation. The spermatic fluid collected with a Pasteur pipette was maintained in a beaker kept on ice until the moment of fecundation. The oocytes, also collect with the aid of a Pasteur pipette, were washed with marine water by using a 0.75 μm pore diameter net and then placed in a beaker with marine water filtered in 0.22 μm *Millipore* membrane.

Fecundation was conducted by mixing 2 mL of the spermatic and oocytes suspension. The egg density sample was estimated by counting eggs with the aid of a *Sedgwick-Rafter* chamber. In each assay, approximately 500 newly fertilized eggs were transferred to each test tube containing the sediment spiked with different concentrations of TCS (7.5–75.0–750.2–7502.0 ng g^{-1}) or IBU (1.5–15.1–150.8–1508.0 ng g^{-1}). Each test tube was a replicate, and four replicates were used for each test concentration of the exposure approaches (SWI, SE). The zygotes were exposed for 48 h at a constant temperature (25 ± 2 °C), photoperiod (16 h light: 8 h dark) and salinity levels (35).

After the exposure period, the larvae were fixed with 40% buffered formaldehyde-borax (pH 7.0). Larvae developed to D-phase were considered normal, whereas those presenting delay or morphological anomalies in their development were considered abnormal. A mean percentage of normally developed larvae were obtained for each tested concentration by quantifying the number of normal developed larvae for the first 100 embryos visualized in each replicate. A positive control assay using sodium dodecyl sulfate (SDS) (concentrations ranging from 0.098 to 1.5 mg.L^{-1}) was conducted in parallel to the sediment assays.

2.3.2. Lytechinus variegatus toxicity assay

The toxicity assays for the evaluation of chronic effects on the embryo-larval development of *L. variegatus* were performed according to the procedures described by the USEPA (1995) with adaptations to this species recommended by the protocol NBR 15350 (ABNT, 2012).

The gametes were obtained by injecting a 0.5 M KCl solution in sexually mature individuals. To collect the oocytes, the females were placed with its aboral surface down in a beaker filled with dilution water. Eventual residues were removed with the aid of a 350 μm pore diameter net. The oocytes were then washed with abundant dilution water and kept in a beaker with 600 mL of the same dilution water. The spermatic fluid, in turn, was collected with a Pasteur pipette directly from the gonopore and disposed of in a dry beaker kept in ice.

A spermatic suspension in a proportion of 0.5 mL of spermatic fluid to 25 mL of dilution water was prepared. Approximately 1.2–2.0 mL of the spermatic suspension was added to the beaker containing the oocytes and the suspension was gently stirred for 10 min to allow fecundation.

The criteria for successful fecundation were a minimum of 80% of fertilized oocytes. In each assay, approximately 300 eggs were added to each test tube containing four different concentrations of spiked sediments with TCS (7.5–75.0–750.2–7502.0 ng g^{-1}) and IBU (1.5–15.1–150.8–1508.0 ng g^{-1}), for a period of 24 h at constant temperature (25 ± 2 °C), photoperiod (16 h light: 8 h dark) and salinity levels (35).

After 24 h of exposure, the assays were finalized by adding 0.5 mL of 40% formaldehyde-borax buffered. The first 100 organisms were analyzed for anomalies or delayed development in a *Sedgewick-Rafter* chamber under an optical microscope. A positive control assay using $ZnSO_4$ (concentrations ranging from 0.22 to 0.80 mg.L^{-1}) was conducted in parallel to the sediment assays as a positive control.

2.3.3. Mytella charruana cytotoxicity assays

The assay for the evaluation of the Neutral Red Retention Time (NRRT) is based on the principle that healthy cells are able to retain the Neutral Red dye longer than stressed cells, since the last may present lysosomes with damaged membrane due to the exposure to xenobiotics (Dailianis et al., 2003) or other stressors.

The experiments were conducted according to the method proposed by Lowe et al. (1995). In each assay, specimens of adult mussels *M. charruana* (n = 12) were exposed to three different concentrations of the analyzed substances (TCS: 0.01–0.08–0.75 ng g^{-1}; IBU: 0.02–0.15–1.51 ng g^{-1}) for 24 h at constant temperature (20 ± 1 °C) photoperiod (16 h light: 8 h dark) and salinity levels (30).

After the exposure period, a 300 μL-aliquot of hemolymph was

taken from each individual with the aid of a syringe containing 300 μL of physiological solution. Then a 50 μL-aliquot of this cell suspension was transferred to a slide containing a Poly-L-lysine solution. The slides were kept in a dark chamber for 15 min for cell fixation. The exceeding hemolymph was removed and then 40 μL of neutral red dye was added over each slide. After 15 min of incubation, the slides were observed using an optical microscope at 400× magnification every 15 min. During this period, the haemocytes were analyzed for structural abnormalities, lysosomal rupture and dye extravasation to the cytoplasm. The test was ended when at least 50% of the examined cells (maximum reading time of 1 min per slide or the evaluation of at least 300 cells) exhibited these characteristics and the NRRT mean value was calculated for each group.

2.4. Data analysis

2.4.1. Toxicity assays evaluation

In order to determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC), the data were firstly analyzed for normality and homogeneity of variance by the Chi-square and Bartlett methods, respectively. Since the data followed parametric assumptions, ANOVA test, with Dunnet's post-hoc test ($\alpha = 5\%$), were employed to compare the means in the treatments groups against the mean obtained in the control group. The statistical analysis was performed with the software TOXTAT 3.5 (Gulley, 1996).

2.4.2. Environmental risk assessment

The environmental risk assessments for TCS and IBU in marine sediments were evaluated by calculating the RQ, according to the protocol established by the European Medicine Agency (EMEA, 2006). The RQ values for aquatic organisms was the ratio between the measured environmental concentrations (MEC) (in the current study, MEC was obtained from sediments from the vicinity of the sewage outfall of Santos Bay) and the Predicted-No Effect Concentration (PNEC) (obtained from the battery of bioassays performed in the current study). The PNECs is the lowest NOEC obtained in the bioassays divided by a factor 10, an expression of the uncertainty of the data collected from the toxicity assays, considering the principle of prevention and precaution (Equation (1)). The classification criteria used was: RQ < 0.1 meaning minimum risk; 0.1 ≤ RQ < 1.0 meaning intermediate risk and RQ ≥ 1.0 meaning high environmental risk.

$$PNEC = \frac{NOEC}{10} \quad (1)$$

3. Results

3.1. Physical and chemical analysis

Sediment grain size and levels of carbonates were quite similar between the reference sediments used in the toxicity assays, and the sediments from the surroundings of the submarine sewage outfall. The level of OM, however, was higher in the sediments affected by sewage. The reference sediment was composed by 7.6% of coarse sand, 27.7% of medium sand, 56.8% of fine sand, 0.7% of very fine sand, 7.2% of silt and clay, 22.1% of carbonates and 0.36% of OM, while the sediment sampled in the study area showed 3.4% of coarse sand, 18.7% of medium sand, 49.0% of fine sand, 20.2% of very fine sand, 8.5% of silt and clay, 18.1% of carbonates and 4.2% of OM.

The linearity and the precision of the analytical methods were evaluated using non-contaminated sediments, which were spiked with different levels of concentration of PPCP. The recovery rates were 88% for IBU and 100% for TCS. The Limit of Detection (LOD), the Limit of Quantitation (LOQ), and the results of the analysis of the samples from SOS are presented in Table 1.

3.2. Ecotoxicological analysis

3.2.1. Embryo-larval development of P. perna and L. variegatus

The results of the embryo-larval development assays with both species were acceptable since the negative control showed at least 80% of normal development and the IC50 48 h estimated for the positive control were within the concentration range reported in the literature for *P. perna* (Zaroni et al., 2005) or were within the upper and lower limit range of the laboratory control chart (for *L. variegatus*). The results of IC50 48 h of SDS for *P. perna* ranged from 1.01 mg L^{-1} (IC ranged from 0.97 to 1.03 mg L^{-1}) to 1.09 (IC ranged from 1.07 to 1.11 mg L^{-1}), while the IC50 24 h of $ZnSO_4$ estimated for *L. variegatus* ranged from 0.50 mg L^{-1} (IC ranged from 0.50 to 0.52 mg L^{-1}) to 0.54 mg L^{-1} (IC ranged from 0.53 to 0.56 mg L^{-1}).

Figs. 2 and 3 show the results of the SWI and SE bioassays, exposed to TCS and IBU, for *P. perna* and *L. variegatus*, respectively. The assays for *P. perna* showed lower values of NOEC and LOEC in the SWI assays compared to the SE assays, for both tested substances, while the values of NOEC and LOEC for *L. variegatus* assays did not show differences between SWI and SE assays, either for TCS or IBU.

3.2.2. Neutral Red Retention Time Assay

After a 24-h exposure, the retention time of Neutral Red in the lysosomes of the haemocytes was significantly diminished (compared to control treatment) in the concentration treatments of 0.08 and 0.75 ng g^{-1} of TCS, and 0.15 and 1.50 ng g^{-1} of IBU in whole sediments (Fig. 4). Fig. 5 shows healthy cells and cells under stress observed in the optical microscope.

3.3. Environmental risk assessment

The PNEC and RQ values estimated for TCS and IBU are shown in Table 2. Both substances (TCS and IBU) are present in Santos Bay's sediment and shows values of RQ well above 1.0, the threshold for high environmental risk.

The RQ estimated for both TCS and IBU did not differ between *P. perna* and *L. variegatus* embryo-larval development for the SWI exposure, but showed a difference in the case of exposure to SE (the risk was higher for *L. variegatus*). Regarding the RQ estimated from the results of the neutral red assay, the values for TCS and IBU were 750 and 75 times higher, respectively, when compared to the RQ values estimated from the embryo-larval development of both tested species.

4. Discussion

The chemical analysis performed with sediments samples from the vicinity of the submarine sewage outfall at Santos Bay demonstrated the occurrence of TCS (15.14 ng g^{-1}) and IBU (49.0 ng g^{-1}) in the same concentration range as reported by previous studies. Data on sediment concentrations range from 2.0 to 400 ng g^{-1} for TCS and 12.8–100 ng g^{-1} for IBU in aquatic environments worldwide (Agüera et al., 2003; Xie et al., 2008; Wilson et al., 2009; Cantwell et al., 2010; Duan et al., 2013; Pintado-Herrera et al., 2013, 2014; Huber et al., 2016). In Brazil, the presence of TCS in superficial freshwater and bottom sediments was previously reported (Montagner et al., 2014; Sousa et al., 2015), but

Table 1
Parameters of Multiple Reactions Monitoring for the negative ion mode, limits of detection, limits of quantification, retention time and the concentrations of TCS and IBU in sediments from SOS.

Compound	Q1	Q3	DP (V)	CE (V)	CXP (V)	Limit (ng g^{-1})		RT	Concentration (ng g^{-1})
						LOD	LOQ		
Triclosan	287.0	34.9 (MIM)	−40	−29	−3	0.42	1.4	1.54	15.14
Ibuprofen	205.0	161.1 159.0	−15 −15	−10 −10	−2 −2	2.12	7.09	4.65	49.0

Q1 (first quadrupole); Q3 (last quadrupole); DP (Declustering Potential); CE (Collision Energy); CXP (Collision Exit Potential); LOD (Limits of detection: Signal/noise = 3); LOQ (Limits of quantification: Signal/noise = 10); RT (Retention time); MIM (Multiple Ion Monitoring). In Q3, in the upper cell is the quantifier ion and the lower cell is the qualifier ion.

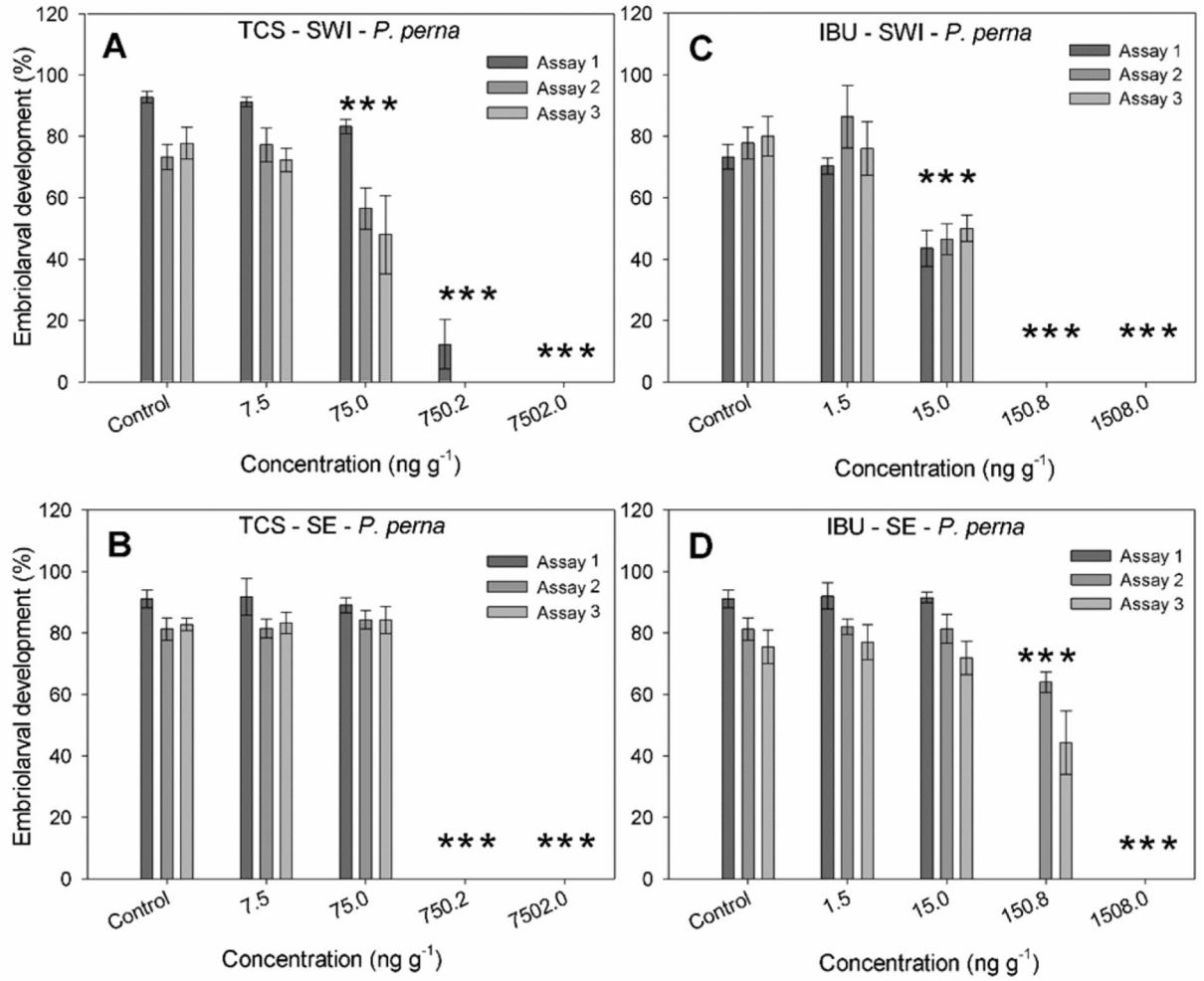

Fig. 2. Results (mean and standard deviation) for *P. perna* embryo-larval development and the NOEC and LOEC for the two substances studied, using both SWI and SE assays. A) SWI exposure to TCS; B) SE exposure to TCS; C) SWI exposure to IBU; D) SE exposure to IBU. Asterisks indicate significant differences compared to control group (ANOVA-Dunnett's test, $p \leq 0.05$).

no data are available about TCS levels in marine sediments. Regarding IBU, the concentration obtained in the current study was close to the concentration reported by Beretta et al. (2014) (14.3 ng g^{-1} in *Todos os Santos* Bay, *Bahia*, NE Brazil).

In the present study, the reference sediment used for the spiking procedure showed toxicity results within the range of acceptance criteria of a negative control. In addition, the toxicity tests showed low standard deviations for the three species tested, demonstrating the efficacy and reproducibility of the sediment homogenization process through the jar-rolling method.

The current results showed that both TCS and IBU, even at low concentrations in sediments, cause adverse effects to marine organisms. These compounds caused toxic effects at environmentally relevant concentrations, even if it is considered only the traditional ecotoxicological assays at individual levels (embryo-larval development). The high risk of TCS in the marine environment due to its toxicity to *P. perna* at environmentally relevant concentrations in marine surface waters was previously reported by Cortez et al. (2012).

The values of PNEC and RQ for both TCS and IBU did not vary

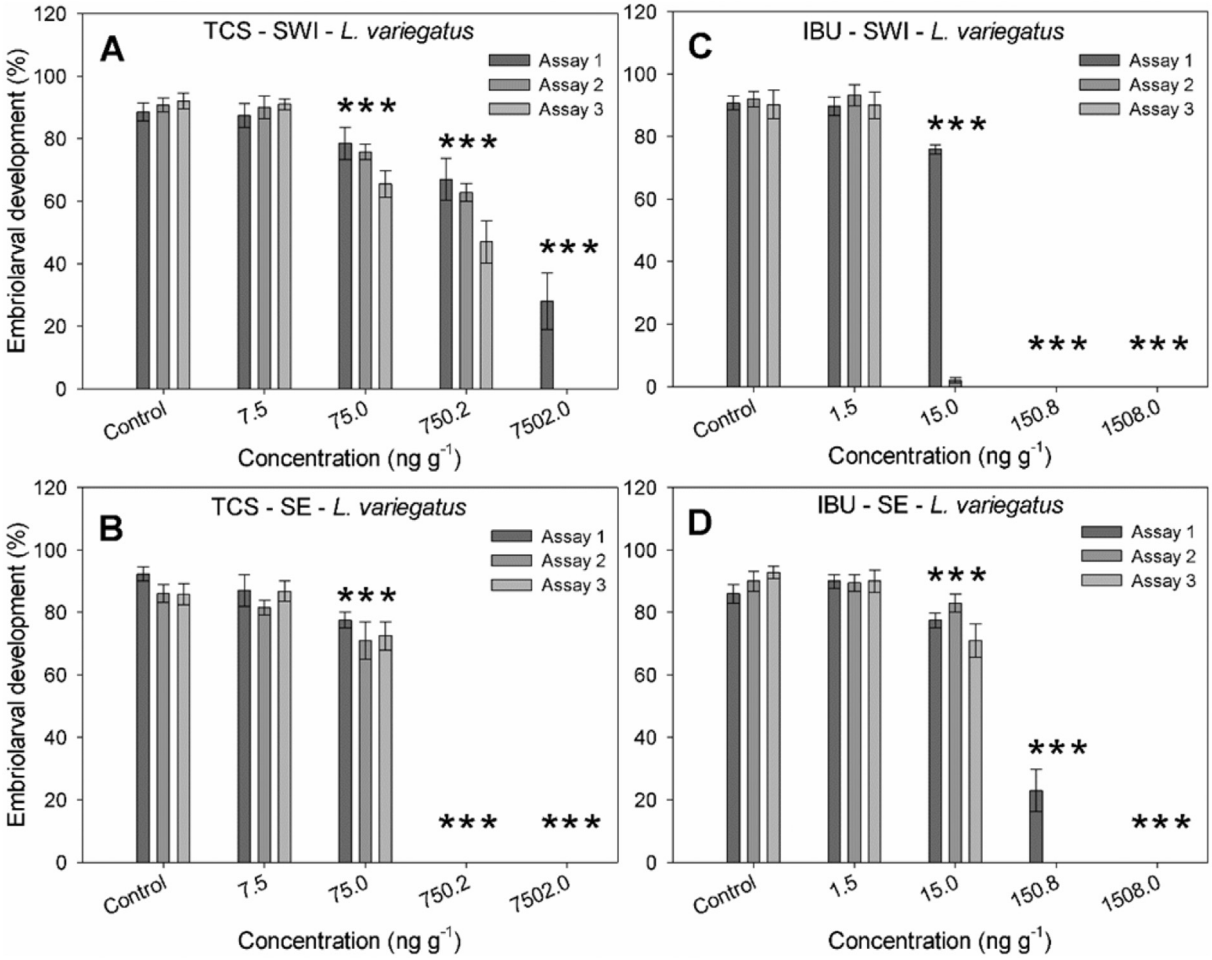

Fig. 3. Results (mean and standard deviation) for *L. variegatus* embryo-larval development, and the NOEC and LOEC for the two substances studied, using both SWI and SE assays. A) SWI exposure to TCS; B) SE exposure to TCS; C) SWI exposure to IBU; D) SE exposure to IBU. Asterisks indicate significant differences compared to control group (ANOVA-Dunnett's test, $p \leq 0.05$).

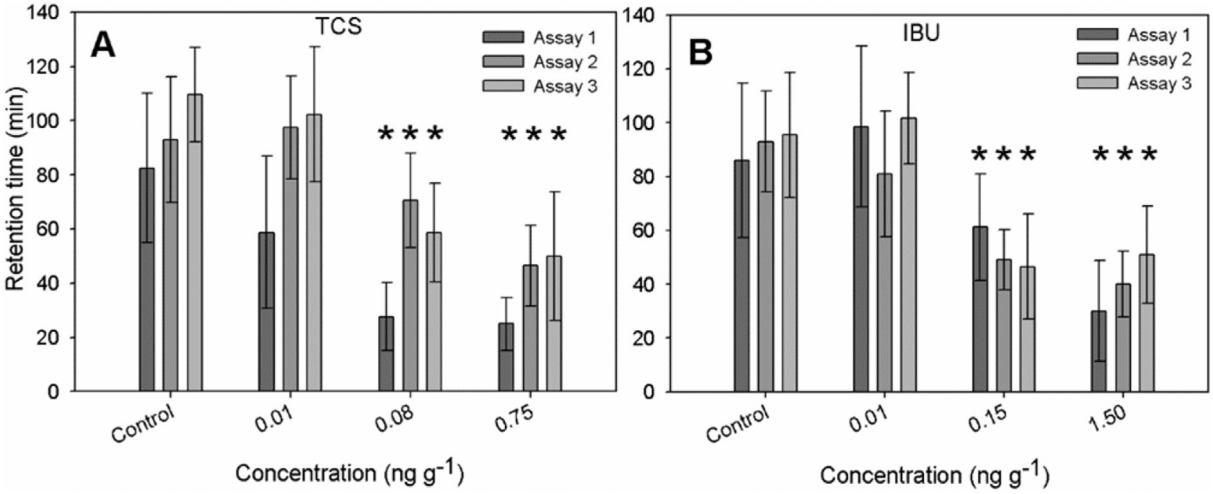

Fig. 4. Results (mean and standard deviation) of the Neutral Red Retention Time Assay for *M. charruana* for both tested substances. A) WS exposure to TCS; B) WS exposure to IBU. Asterisks indicate significant differences compared to control group (ANOVA-Dunnett's test, $p \leq 0.05$).

between the test-species in the sediment-water interface exposure. This situation happened since the range of concentrations tested and the NOEC were the same for both test-species. Consequently, the estimated PNEC and RQ showed identical values for the tested species.

Identically, no differences for PNEC and RQ were found between

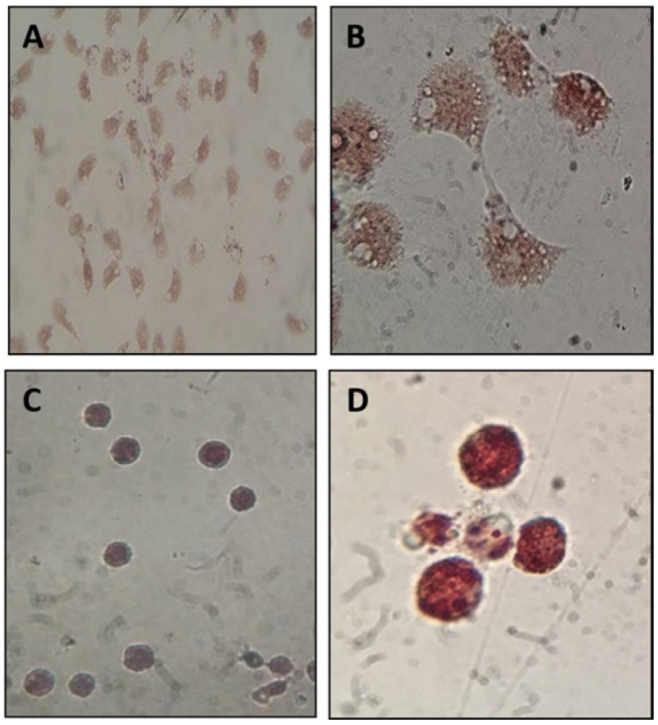

Fig. 5. Healthy and stressed cells of *M. charruana* mussel observed under an optical microscope. A) healthy cells (100x); B) healthy cells (400x); C) cells under stress (100x); D) cells under stress (400x).

SWI and SE for *L. variegatus*, suggesting that sediment resuspension does not alter substantially the toxicity of the tested compounds. However, the results for *P. perna* showed that PNEC and RQ for the SWI exposure were higher than the values estimated for the SE exposure. It could be related to the bioavailability of the compounds in the sediments, the elutriate and the water column, as well as the equilibrium constant and the acid dissociation constant of the compounds (pKa). In the SE exposure, the sediment was stirred in marine water for 30 min and, thereafter, the supernatant was used for the organisms' exposure. In the SWI exposure, organisms were exposed only after 24 h of interaction between water and sediment, and the system was kept throughout the organisms' exposure (48 h) (static condition). Thus, in the SWI system the sediment interacts for longer time with water, thus permitting greater dissolution of the compounds with low pKa compared to the SE system. Therefore, the toxic effects of TCS (pKa = 7.9) and IBU (pKa = 4.91) were higher in SWI exposure, and may be related to the pKa values of these compounds. The current results reinforce the importance of using different tests to evaluate toxicity in test organisms and exposure times, especially in areas like Santos Bay where the sediments are prone to resuspension not only due to natural factors (e.g. tidal currents, storms), but also due to intense port activities, such as the turbulence caused by large vessels and dredging activities.

To our knowledge, few studies have assessed the effects of spiked sediments with PPCP, especially on marine or estuarine benthic organisms. Perron et al. (2012) showed acute toxicity of TCS spiked sediments on concentrations significantly higher than the one obtained in the present study. After 7-days exposure, the lethal concentration to amphipoda *Ampelisca abdita* and mysid *Americamysis bahia* were 303 and 257 μg g^{-1}, respectively. However, concerning IBU, Maranho et al. (2015a) reported toxic effects in the same range of concentrations observed in this study. They performed a battery of bioassays to assess toxicity in sediments using marine organisms commonly used in the environmental assessment. *Vibrio fischeri* bioluminescence inhibition assay showed a concentration effect (IC50) of 100.6 ng g^{-1} and algae growth inhibition (*T. chuii*) at a concentration of 5 ng g^{-1}. Maranho et al. (2014) also reported enzymatic alterations on polychaete *H. diversicolor* after 14-d exposed to concentrations ranging from 0.5 to 50 ng g^{-1}.

Although never used before in toxicity tests, *M. charruana* can be an excellent sentinel species for monitoring and evaluation of marine sediments quality, especially for estuarine areas. This bivalve mussel is commonly found in tropical and sub-tropical estuaries in the South American Atlantic coast, living buried in sediments and the current results showed that the species is sensitive to PPCP in cytotoxic tests, suggesting that it can be a suitable bioindicator for studies about the environmental quality of such compounds.

Considering the sub-individual level assays, both TCS and IBU presented negative effects on lysosomal membrane stability of *M. charruana* haemocytes at environmentally relevant concentrations in marine sediments (0.08 ng g^{-1} and 0.15 ng g^{-1}, respectively). Although the NRRT assay is a sub-individual assay, disturbs on the lysosomal membrane can trigger effects at higher biological levels of organization. For example, cellular stress caused by parental generation exposure to xenobiotics can affect the reproductive success of these populations on a long-term, since the embryo-larval development of some invertebrates (e.g. sea-urchin and mussels, among others) is dependent on the energy released by the yolk by means of the action of the lysosomes (Ringwood et al., 2004). Moore et al. (2006) and Pereira et al. (2014) showed associations between NRRT responses and macrobenthic diversity in estuarine environments, suggesting a good ecological relevance of this biomaker. The use of biomarkers together with responses at higher levels of biological organization is important for more accurate environmental risk assessments.

Toxicity effects observed here could be explained by the mechanism of action of TCS and IBU. TCS toxicity is mediated at least in part through its membranotropic effects, affecting the functional integrity of cell membranes (Villalaín et al., 2001), as observed in the NRRT assay. IBU, in turn, stabilizes lysosomal membranes in mammals at therapeutical levels (which accounts for its anti-inflammatory effect) (Smith et al., 1976; Flynn et al., 1984), but it can cause significant reduction of lysosomal

Table 2
PNEC and RQ for TCS and IBU during the exposure of *P. perna* and *L. variegatus* to SWI and SE assays and *M. charruana* exposed to WS assays.

Substance	*P. perna*				*L. variegatus*				*M. charruana*	
	SWI		SE		SWI		SE		WS	
	PNEC	RQ	PNEC	RQ	PNEC	RQ	PNEC	RQ	PNEC	RQ
TCS[a]	0.75	20.18	7.5	2.01	0.75	20.18	0.75	20.18	0.001	15 140
IBU[b]	0.15	326.6	1.51	32.4	0.15	326.6	0.15	326.6	0.002	24 500

[a] MEC = 15.14 ng g^{-1}.
[b] MEC = 49.0 ng g^{-1}.

membrane stability at high concentrations (Phillips and Muirden, 1972). The reduction in lysosomal membrane stability after exposure to pharmaceuticals (including IBU) has also been observed in non-target organisms. Aguirre-Martínez et al. (2013a) showed effects in the membrane stability of lysosomes of marine organisms, such as the clam *R. philippinarum* after a 7-d exposure to waterborne IBU (15 μg L^{-1} to 50 μg L^{-1}). The crab *Carcinus maenas* showed a 50% reduction of lysosomal membrane stability compared with control individuals after 28-d of exposure of IBU (5 μg L^{-1}), carbamazepine (1 μg L^{-1}), and novobiocin (1 μg L^{-1}) in water (Aguirre-Martínez et al., 2013b). Milan et al. (2013) showed a decrease in antioxidant enzyme activity in clams *R. philippinarum* exposed to IBU. According to Maranho et al. (2015c), variations of the antioxidant defense may have several consequences, including alterations in the stability of the membranes of lysosomes. Concerning TCS, Cortez et al., (2012) observed effects in the lysosomal membrane stability of the marine mussel *P. perna* exposed to 1.2 μg L^{-1} in seawater.

Thus, TCS and IBU, acting directly on cell membranes by membranotropic effects and consequent disruption of lysosomal membranes, showed increased cytotoxic effect when compared to traditional assays for chronic effects evaluation. These results reinforce the need for more studies towards the mechanisms of action for these compounds to obtain more sensitive responses (Martin-Diaz et al., 2009; Cortez et al., 2012).

The establishment of RQ both at an individual level as well as at sub-individual level has demonstrated that TCS and IBU at Santos Bay presented a high environmental risk to benthic invertebrates. In the current study it was used the Measured Environmental Concentration and the PNEC was derived from toxicity assays, which confers more reliability to the PNEC and the RQ compared to other approaches (e.g. PNECs obtained from toxicity data that have considered the equilibrium partitioning method) (Hernando et al., 2006; Nie et al., 2015). Bouissou-Schurtz et al. (2014) found large differences of RQ values for IBU (e.g.) when they compared the PEC/PNEC index (RQ = 600.0) and MEC/PNEC index (RQ = 1.9). Therefore, it is important that environmental risk assessments of PPCP in sediments consider the use of MEC as well as the establishment of PNEC from toxicity assays at different levels of biological organization.

Recently, the European Commission, in the scope of Directive 2013/39/EU, has established a list of priority for chemicals which constitute a significant risk to the aquatic environment (EC, 2013). Moreover, the Commission established a watch list of substances, which includes Diclofenac, 17-beta-estradiol and 17α-ethinylestradiol, in order to gather monitoring data of environmental concentrations and toxic effects for the determination of appropriate measures to address the risk posed by those substances. According to the results obtained in this study, TCS and IBU should be included in the next review of the priority list of toxic substances.

5. Conclusion

Considering the growing need for environmental risk assessment to PPCP in marine sediments, this study presents the first data based on both measured environmental concentrations and PPCP spiked sediments in Latin America. Both triclosan and ibuprofen presented a high environmental risk and should be considered in future legislation on environmental management and waste policies as well as in wastewater treatment, in order to minimize possible environmental impacts. The marine benthic bivalve *Mytella charruana* proved to be a suitable bioindicator for studies on the environmental quality of tropical and subtropical regions, and it is an excellent alternative sentinel species for monitoring of marine and estuarine sediments quality.

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