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Metal toxicity assessment by sentinel species of mangroves: *In situ* case study integrating chemical and biomarkers analyses

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ABSTRACT

Globally, there is a lack of knowledge about tropical ecotoxicology dealing with the potential impact of metal contamination in mangrove ecosystem. This habitat is considered a nursery for several animal species, among them the "uçá"-crab (*Ucides cordatus*), known as a key species due to its biological and economical importance. This study evaluated the association involving metal contamination (Cd, Cu, Pb, Cr, Mn and Hg) in water, sediment, red-mangrove vegetation (*Rhizophora mangle*) and tissues of uçá crab, together with its geno-cytotoxic responses, based on micronucleated hemocytes frequency and the retention time of neutral red in lysosomes. We assessed six mangrove areas with distinct pollution levels in São Paulo State, Brazil, where the water and sediment contamination by metals were associated with accumulation of these pollutants in biotic compartments (mangrove leaves and crab). In *U. cordatus,* metal accumulation was best explained by metal concentration found in leaves of *R. mangle* than in the water or sediment, indicating that feeding drives metal exposure in this organism. Mercury (Hg) concentration in sediment, copper (Cu) concentration in hepatopancreas of *U. cordatus* and lead (Pb) in water and green leaves of *R. mangle* showed a significant correlation with genotoxic impact in *U. cordatus*. However, copper concentration (in green/senescent leaves and hepatopancreas) and lead (in sediment), were the major metals affecting lysosomal membrane integrity. Therefore, representatives of all compartments were associated with cyto and genotoxicity in this species, thus requiring a holistic approach to issues related to sublethal damage. Probability estimates of cytogenetic impacts related to metal concentration in abiotic compartments (significantly correlated with known biomarkers: Hg in sediment; and Pb in water and sediment) are also presented. Our results highlight the need for environmental restoration of mangroves areas contaminated with metals, responsible for cytogenetic injuries and revealing a pre-pathological condition in this sentinel species, in addition to ecological disturbances.

1. Introduction

Tropical ecosystems account for most of the world's biodiversity (Barnes et al., 2014), even though the focus of ecotoxicological studies are, historically, almost exclusively on temperate environments (Lacher and Goldstein, 1997; Sueitt et al., 2015). This lack of knowledge comes from the increasing and constant threat of toxic substances released into the environment, and water bodies, which causes negative impact for tropical aquatic organisms (Peters et al., 1997; Harford et al., 2015).

Metals are among the known pollutants with the highest degree of toxicity and persistence (Rainbow, 1997, 2007; Ahearn et al., 2004; Rainbow and Black, 2005; Luoma and Rainbow, 2008), attracting interest at a global level, due to their large impact and potential risk to the biota. Metals are difficult to break down, accumulate in animal and vegetal tissues, and are magnified in trophic chains (Ahearn et al., 2004; Rainbow, 2007; Vilhena et al., 2012). There are metals considered essential (such as Cu, Cr and Mn), important for animal metabolism, although they become toxic if concentrations are high. Non-essential metals (such as Hg, Pb and Cd), on the other hand, cause toxicity even at small concentrations (Eisler, 2010). In all instances, and

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depending on the dosage, the presence of metals is of concern because of their mobility in water, their persistence in sediments (Luoma and Rainbow, 2008; Vilhena et al., 2012) and their bioavailability and toxicity in biota (Ahearn et al., 2004; Rainbow, 2007). Even if accumulation of these elements serves as an important parameter for quantifying the level of contamination on natural environments (Rainbow, 2007; Luoma and Rainbow, 2008), recent criteria on sublethal biological disturbances have been considered as the best way to monitor natural environments (Pereira et al., 2011, 2014; Duarte et al., 2016).

There are few "*in situ*" studies in the literature, and it is well known that pollution by metals can generate biological disturbances in organisms, which can be identified and quantified by specific biological assays known as biomarkers (Monserrat et al., 2007; Amiard-Triquet, 2011; Amiard-Triquet et al., 2013b; Pereira et al., 2014). Biomarkers can detect early sublethal disturbances that could potentially impact the entire population or communities (Amiard-Triquet et al., 2013b; Duarte et al., 2016; Pinheiro et al., in preparation). Genetic and physiological disturbations are one of the first sublethal modifications observed in organisms exposed to metals (Otomo and Reinecke, 2010; Amiard-Triquet et al., 2013b; Pinheiro and Toledo, 2010; Pinheiro et al., in preparation). Thus, to estimate these alterations, many authors have used the technique of micronucleated cells, expressed in MN‰ (Scarpato et al., 1990; Brunetti et al., 1988; Al-Sabti and Metcalfe, 1995; Burgeot et al., 1995; Hoshina et al., 2008; Collier et al., 2013; Pinheiro et al., 2013), as well as the neutral red technique, quantified through the retention time of the neutral red (NRRT) in lysosomes (Lowe et al., 1995; Svendsen et al., 2004; Daguano et al., 2007). These techniques have large ecological importance for populations of affected areas (Bonassi et al., 2000; Neri et al., 2003; Duarte et al., 2016), having been applied in freshwater (Falfushynska et al., 2014; Taylor et al., 2017), estuarine (Pereira et al., 2014; Duarte et al., 2016) and marine environments (Catharino et al., 2008; Buratti et al., 2012; Wyatt et al., 2014).

The use of biological models (bioindicators) may reveal "sentinel" species, named so because of early metal toxicity detection in natural environments, some of them having an important ecosystem function (Beltrame et al., 2010, 2011; Pereira et al., 2014). The land crab *Ucides cordatus* is semi-terrestrial and endemic of mangroves areas and is an example of a sentinel species (Fiscarelli and Pinheiro, 2002; Nordhaus et al., 2009), standing out as a testimony species of environmental quality (Pinheiro et al., 2012, 2013; Duarte et al., 2016; Ortega et al., 2016). *Ucides cordatus* is consumed by fish, birds and mammals, including man (Fiscarelli and Pinheiro, 2002), and is responsible for metal biomagnification in the mangrove trophic chain. In addition, they have an intimate relationship with the components of the environment they inhabit, namely: 1) water, through contact and ingestion, participating in various physiological and metabolic processes, among them breathing (Pinheiro et al., 2012); 2) sediment, through contact and ingestion, digging galleries and ingesting part of the sediment during feeding (Nordhaus et al., 2009); and 3) mangrove leaves, used as food by this species, mainly the red-mangrove *Rhizophora mangle* (Pinheiro et al., 2013; Christofoletti et al., 2013). Moreover, the species has a relatively long life cycle, reaching its maximum size at 10 years of age (Pinheiro et al., 2005). The uçá-crab is also a food source, particularly by coastal populations, and has a wide distribution in the mangroves of the Western Atlantic (Melo, 1996), many of which are subject to anthropic pressure, mainly in southeastern Brazil (Abessa and Ambrozevicius, 2008; Pinheiro et al., 2013, 2017; Duarte et al., 2016).

Duarte et al. (2016) demonstrated the effectiveness of uçá-crab as an indicator species for determining the conservation status of mangrove areas employing biological responses and their linkage to contamination. The authors addressed a multi-level biological approach (biomarkers, condition factors and crab density) and their strong association with the local contamination (determined *via* information reported in scientific and technical literature about metals, organochlorine pesticides, polychlorinated biphenyl and polycyclic aromatic hydrocarbon) and solid waste volume. This previous study also described that density of crabs was correlated with subcellular biomarkers, provided the normal baseline values for the biomarkers (frequency of micronucleated cells, MN‰ < 3; neutral red retention time > 120 min) and categorized three levels of human impacts in mangrove areas (PNI, probable null impact; PLI, probable low impact; and PHI, probable high impact). However, the group(s) of contaminants responsible for cyto-genotoxicity effects observed by Duarte et al. (2016) are still unknown. According to Eisynk et al. (1988), CETESB (2001) and Pinheiro et al. (2012, 2013, 2017), metals are the most historically threatening contaminants in state of São Paulo mangroves of state of São Paulo. Therefore, the present study aims to test the hypothesis that there is a significant relationship between cyto-genotoxicity effects and levels of metal contamination in abiotic (water and sediment) and biotic compartments (green/senescent leaves of *R. mangle* and tissues of *U. cordatus*). For this purpose, first, we assessed whether abiotic contamination by metals is linked to bioaccumulation and whether the association involving the presence of metals (Cd, Cu, Pb, Cr, Mn and Hg) in all compartiments is linked to sublethal responses recorded in a sentinel species of Western Atlantic mangroves.

2. Materials and methods

2.1. Study area

In Brazil, the coastal region of São Paulo State represents a contrasting scenario due to the presence of preserved areas, together with areas subjected to significant environmental impact (Pinheiro et al., 2008). In order to study the relationship between metals and sublethal disturbances, six mangrove areas of the State of São Paulo (Bertioga, BET; Cubatão, CUB; São Vicente, SAV; Iguape, IGU; Juréia, JUR; and Cananéia, CAN) were studied, based on their anthropic historical pressure, particularly with respect to contamination by metals (Eisynk et al., 1988; CETESB, 2001; Pinheiro et al., 2013). Therefore, the present study was developed in the same areas previously studied by Duarte et al. (2016) and Pinheiro et al. (in preparation), which represent the vast majority of the state's mangrove area (99%). Six mangrove areas have been established (Fig. 1, with local icons characterizing each mangrove area), three of them located on the central coast (BET, CUB and SAV) and extra three established on the southern coast (IGU, CAN and JUR - the latter an ecological station). Each mangrove area includes three subareas (replicates) that are similar in tree composition (>50% *Rhizophora mangle*), vegetation structure (tree height >5 m; and diameter at breast height >10 cm), flood tide height (>30 cm) and productivity (leaves available on the sediment $>2\ g\ m^{-2}$).

Located on the central coast of São Paulo, the **Bertioga** Estuary (BET) receives domestic untreated sewage and effluents from a public garbage dump as its main source of pollution. Even so, the estuary is used for ecotourism and sport fishing (Eichler et al., 2006). **Cubatão** (CUB) is considered one of the most polluted regions in the world; in recent years, it has become the most important Brazilian industrial hub, formed by 23 industrial complexes, 111 factories, and more than 300 pollution sources. Home to the largest port in Latin America, it also experiences transit of large vessels carrying several types of chemicals (Martins et al., 2011; Pinheiro et al., 2012, 2013). Located within the same estuarine complex there is **São Vicente** (SAV), showing substantial human activity, illegal slums, a lack of wastewater treatment and solid waste collection (CETESB, 2007; Kirschbaum et al., 2009; Cordeiro and Costa, 2010; Mello et al., 2013), and intensified by 11 industrial sources of pollution. These areas, therefore, have a history of substantial human impact, with records of contamination by various xenobiotics, including metals (Azevedo et al., 2009; Pinheiro et al., 2012, 2013).

The mangrove areas on the southern coast of São Paulo are

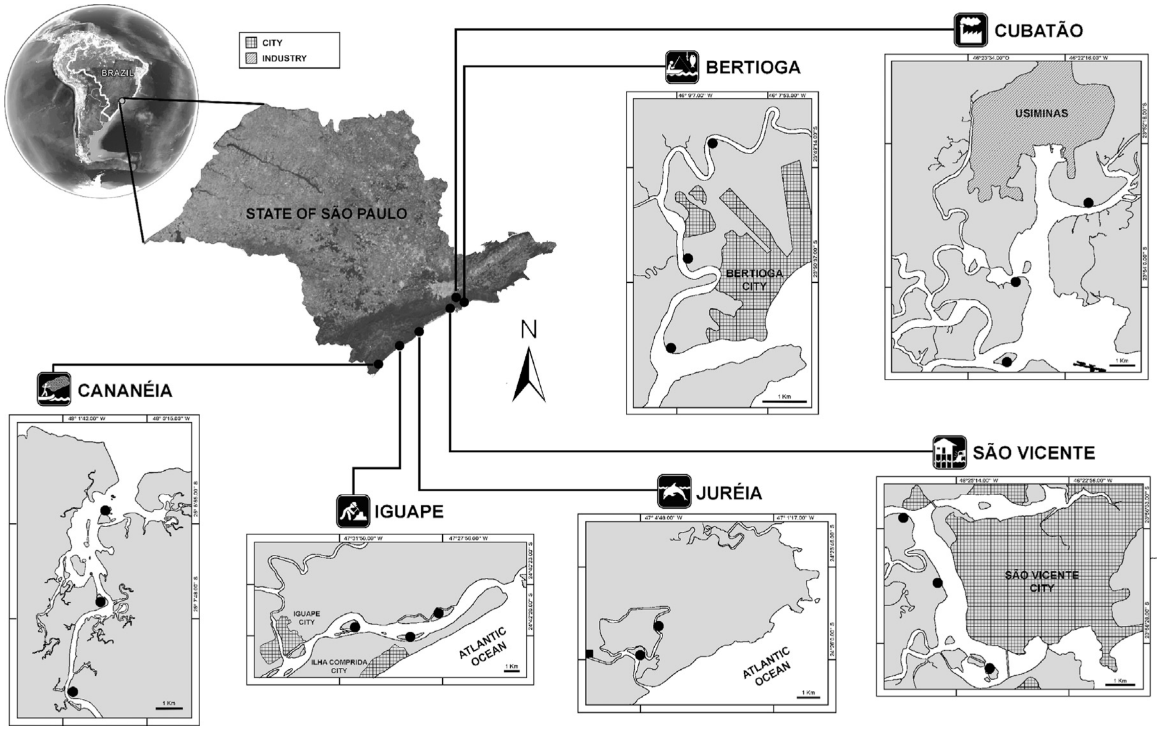

Fig. 1. Location of the six mangrove areas sampled in the state of São Paulo (Brazil), represented by 18 subareas located in the central and southern coasts (Source: Satellite images from Google Earths® redrawn by Gustavo Pinheiro). Each mangrove area is represented by a symbol that informs the principal anthropic impact or its main characteristics of environmental condition.

approximately 80–200 km away from the mangrove areas found on the central coast. The southern coast contains estuaries containing well conserved areas, though some of these areas have already exhibited effects of indirect anthropogenic influence. The entire estuarine system, which includes Cananéia, Iguape, and Juréia, is located within a single environmental protection area (EPA) that also includes an expressive part of the state of Paraná coast. Together, these landmasses form the Lagamar Complex, one of the largest estuarine complexes in Brazil, and recognized in 1991 as the first Brazilian Biosphere Reserve by UNESCO. It covers 3287 km^2 and includes other interconnected basins (ISA, 1998). Located in the northeastern portion of this complex there is an area known as **Iguape** (IGU), which until the early 1990s received significant waste discharge from rich heavy metal mining in the Ribeira River approximately 5.5 metric tons per month of Pb, Zn, Ag, As, Cd, Cu, and Cr, according to Eysink et al. (1988) and CETESB (2000, 2007). The area known as **Cananéia** (CAN) is located 50 km to the southwest. It includes preserved mangroves and excellent environmental quality (Duarte et al., 2014, 2016). The mangroves in **Juréia** (JUR) are located approximately 50 km northeast of Iguape, and are located inside the Juréia-Itatins Ecological Station (EEJI). The human population is smaller in this region and depends on traditional extractive activities that are free of contaminants (Souza and Barrela, 2001; Marques and Duleba, 2004; Pinheiro et al., 2013). The station is therefore effective in protecting local biodiversity (Bruner et al., 2001). In summary, **Cananéia** and **Juréia** are considered pristine estuarine regions of São Paulo State (Azevedo et al., 2009; Duarte et al., 2016).

2.2. Collection of samples from abiotic and biotic compartments (water, sediment, U. cordatus tissues and R. mangle leaf stages) and methods for metal quantification

All samples were collected in winter/2011 according to the protocol described in Pinheiro et al. (2012, 2013). In each mangrove subarea, three water samples (100 mL) obtained from the *U. cordatus* burrows were collected by suction using a silicone hose and stored in labeled polyethylene bottles. Thus, 54 samples of water (6 areas *vs.* 3 subareas *vs.*3 samples) were obtained. In the same mangrove subareas, three sediment samples were also collected (500 g) from a depth of up to 10 cm near the uçá crab burrows, but in a zone without bioturbation signs (Pülmanns et al., 2014). Each of 54 samples had their roots previously removed with plastic sieves and placed in labeled polyethylene flasks. The choice of crab tissues as well as the tree foliar stages for metals quantification was determined based on the preliminary screening performed by Pinheiro et al. (2012). Considering this bioaccumulation study, the hepatopancreas (midgut gland with detox functions in crustaceans) and muscle (source of human food) were sampled, while for the red mangrove trees it was selected the green and senescent leaves (preferential food items for the crab, according to Christofoletti et al., 2013).

In each mangrove subarea, 20 leaves of *R. mangle* were sampled for each foliar maturation stage (green mature and pre-abscission senescent) according to Pinheiro et al. (2012). Leaves were removed with pruning shears and placed in labeled plastic bags for transport to the laboratory. Leaves were immediately washed (1st washing in running water; water with 5% neutral detergent; 2nd washing in running water; solution of distilled water saturated with HCl; and a large volume of distilled water), to prevent atmospheric contamination of these tissues.

U. cordatus specimens were hand-caught inside their galleries by the "braceamento" method (directly by inserting the crab-catchers arm into the gallery) or with a "redinha" (an artisanal trap constructed by crab-catchers with nylon cord), according to Fiscarelli and Pinheiro (2002). For purposes of standardization, metals in the crab tissues were analyzed only for intermolt adult males (CW, carapace width > 60 mm) (Pinheiro et al., 2012), avoiding any effect of sex, molting and life stage, as previously reported for other decapod crustaceans by Chou et al. (2000) and Chen et al. (2005). The animals collected underwent biometric procedures, including body size (CW) measurements using precision calipers (0.01 mm) and total wet weight (WW) measurements using a precision scale (0.01 g). Captured specimens (n = 54) were placed in coolers with bags of ice and transported to the laboratory, where they were brushed to remove mud. Captured adult crabs (measured with 0.05-mm precision calipers), were dissected with sterilized scissors and tweezers to remove samples of muscle and hepatopancreas, in standardized locations: (i) muscle of the chelar propodus, known for high metal accumulation verified by Chen et al. (2005); and (ii) middle lobe of hepatopancreas, which has a particularly high metabolic rate (Mourente, 1996). Tissue samples (n = 108) were placed in Eppendorf vials, kept frozen (− 20 °C) and transported cold to the CEATOX Laboratory, as described in Pinheiro et al. (2012).

Each sample (water, sediment, *U. cordatus* tissues and leaf stages of *R. mangle*) was analyzed for six metals (Cd, Cu, Pb, Cr, Mn and Hg) using the mineralization method with HNO_3 at 65%, according to Basset et al. (1981). Analyses were optimized by hollow cathode lamps (LCO), according to the metallic element analyzed, and samples were read using a GBC-932 AA atomic absorption spectrophotometer (Athanasopoulos, 1994). The equipment was calibrated using metal stock solutions (1000 ppm). The metal concentration of each sample is expressed in micrograms (µg/g) or nanograms (ng/g) of metal per gram of dry tissue, with minimum detected concentration represented as µ/g (Cd < 0.01; Cu and Mn < 0.02; Pb and Cr < 0.05) and ng/g (Hg < 0.001).

2.3. Diagnosis of sublethal damage in U. cordatus species: genotoxicity via Micronucleus Test (MN‰) and cytotoxicity according to Neutral Red Retention Time (NRRT)

During winter/2013, hemolymph samples were collected for analysis of MN‰ and NRRT, corresponding to 10 specimens/mangrove subarea. The methods are described in detail in Duarte et al. (2016).

2.4. Statistical analysis

Excel and R 2.13.0 were used to design the figures and perform the statistical analyses (Ihaka and Gentleman, 1996). The average concentration of the six total metals (Cu, Cd, Cr, Mn, Pb and Hg) quantified in the biotic and abiotic compartments of the 18 subareas were gathered in six different matrices of data, namely: **Matrix 1**, water contamination; **Matrix 2**, sediment contamination; **Matrix 3**, environmental contamination (water, W; and sediment, water, W; and sediment, S) (Matrix 1 + Matrix 2); **Matrix 4**, red-mangrove bioaccumulation (green mature leaves, GL; and senescent pre-abscission leaves, SL); **Matrix 5**, crab bioaccumulation (hepatopancreas, H; and musculature, M); and **Matrix 6**, biota accumulation (Matrix 4 + 5);

Some matrices were correlated using Mantel test based on the Spearman correlation coefficient (r) (Legendre and Legendre, 1998) in order to infer and presuppose the possible bioavailability (biotic intake from environmental contamination) and transfer (exposure routes between compartments) of the quantified metals. These analyzes were performed using Vegan package (Oksanen et al., 2013). It was assumed that the metal bioaccumulation recorded in hepatopancreas and muscle (Matrix 5) was the response variable, while metals in water (Matrix 1), sediment (Matrix 2) and leaves of *R. mangle* (Matrix 4) were determined as explanatory variables (metals sources for uçá crab). Moreover, the environmental contamination by metals (Matrix 3) was correlated with biota contamination (Matrix 6).

Spearman correlation analyses (which does not require the assumption that the relationship between the variables is linear) were performed using the average of quantified metals (six metals concentrations in six compartments) *vs.* sublethal biomarkers average (MN‰ e NRRT). We analyzed 72 possible relationships (2 biomarkers *vs.* 6 metals *vs.* 6 compartments), whose Spearman coefficients were compared using n-2 degrees of freedom (df = 94) and 5% statistical significance (Zar, 1999). Scatter plots represented relationships with significant linear correlation coefficients between biomarkers and metals in the compartments.

Based on the results of Spearman correlations, only the significant correlations between metal contamination in the abiotic compartments (water and sediment) and the sublethal crab responses (MN ‰ and NRRT) were selected, in order to estimate the probabilities of genocytotoxic impact of metals that were responsible for physiological and genetic injures. The results of cumulative relative frequency for the biomarkers were adjusted to a non-parametric sigmoid model, adapted from Hovgård and Lassen (2000), by estimating least squares between metals concentrations and sublethal damages. It was calculated using the following equation:

$$P = 1/(1 + e^{-r(C - CGC50\%)})$$

Where P is the probability of physiological or genetic impact; r is the slope; $CGC_{50\%}$ is the metal concentration where the probability of genotoxic (CG) or cytotoxic (CC) impact is 50%; and C, is the metal concentration. The metals concentration in the water and sediment (x-axis) were plotted according with the expected genotoxic and cytotoxic damages (y-axis), represented by micronuclei frequency and neutral red retention time.

3. Results

Copper was detected in all abiotic-biotic compartments, and for all mangrove areas, and the same occurred for manganese. Lead was detected in 67% of the abiotic-biotic compartments (water, sediment and leaves) only in Bertioga and Cubatão, and was not detected in *U. cordatus* tissues (hepatopancreas and muscle). Chromium was detected for all mangrove areas and was present in 67% of the abiotic-biotic compartments, but was undetected in water samples and crab muscle. Cadmium was present, like lead, in 50% of the abiotic-biotic compartments, except in water samples and crab tissues. Similar association occurred with mercury, being detected in 50% of compartments (sediment, hepatopancreas and musculature) and in all of the studied areas.

The animals used for sublethal damage assessment had an average carapace width of 77.41 mm (± 5.53) and an average weight of 210.7 g (± 38.92). Genotoxicity results (MN‰) in mangroves were in the following order: SAV > CUB = BET = IGU > JUR = CAN. A similar order for cytotoxicity results (NRRT), were as follows: CUB > BET = SAV ≥ IGU > CAN > JUR.

Mantel tests did not indicate a significant correlation (r = 0.15; p > 0.05) for water contamination by metals (Matrix 1) and its accumulation in the tissues of *U. cordatus* (Matrix 5), the same occurring between this last matrix and sediment contamination (Matrix 2) (r = 0.04; p > 0.05). However, there was a high positive and significant correlation (r = 0.72; p = 0.0001) between environmental contamination (Matrix 3) and biotic contamination (Matrix 6), possibly evidencing the transfer of metals from the abiotic to the biotic compartments. Also, a positive and significant association (r = 0.29, p = 0.04) was confirmed between the bioaccumulation of metals in leaves of *R. mangle* and uçá crab (Matrix 4 *vs.* Matrix 5), inferring that feeding is the main route for metal intake for *U. cordatus*.

Table 1 illustrates the correlations verified between metal concentration in the environmental compartments and the sublethal damages (MN‰ and NRRT) recorded for *U. cordatus*. From the 72 possible correlations, only 13.9% (n = 10) presented significant Spearman correlation coefficients (p < 0.05), five of them for each biomarker (05 correlations with Cu; 03 with Pb; and 02 with Hg). Fig. 2 presents significant linear correlation (p < 0.05 and determination coefficients [R2] superior to 0.70). From these 10 significant Spearman correlations, only 2 involving the metal Pb (in senescent leaf and sediment) were related linearly (p < 0.05) to sublethal damage. There was no dose-response relationship involving Mn, Cd and Cr.

The mean concentration of Cu (in hepatopancreas of *U. cordatus*), Pb (in water and senescent leaves of *R. mangle*) and Hg (in sediment) were significantly associated with uçá crab genotoxicity (MN‰). In addition, there was a significant negative correlation between the cytotoxicity

Table 1
Spearman's correlations (r) considering the empirical points of the relationship involving mean frequency of micronucleus (MN/1000 cells) and neutral red retention time (NRRT minutes) in *U. cordatus vs.* concentration of each metal (Cu, Cr, Mn, Hg, Pb and Cd) in the environmental compartments studied (WA = water; SE = sediment; GL = green and SL = leaves of *R. mangle*; MU = muscle and HE = hepatopancreas of *U. cordatus*). The correlations highlighted in bold and with an asterisk (*) were significant ($p < 0.05$). The dashes (-) represent the relationships that were not possible to analyze due to incomplete data or low sample size (less than 3).

Metal	Compartment	MN (‰)	NRRT (min.)
Cu	WA	−0,15	0.36
	SE	0.36	−0.57
	GL	0.41	**−0.75***
	SL	0.33	**−0.64***
	MU	0.16	**−0.54***
	HE	**0.70***	**−0.58***
Cr	WA	–	–
	SE	−0,29	0.40
	GL	–	–
	SL	–	–
	MU	–	–
	HE	0.39	0.01
Mn	WA	–	–
	SE	−0.16	0.47
	GL	0.11	0.40
	SL	0.12	0.34
	MU	0.41	−0.03
	HE	0.17	0.04
Hg	WA	–	–
	SE	**0.68***	−0.41
	GL	–	–
	SL	–	–
	MU	−0.17	0.40
	HE	0.26	0.05
Pb	WA	**0.81***	−0.02
	SE	−0.40	**−0.94***
	GL	**0.72***	0.25
	SL	**0.98***	0.08
	MU	–	–
	HE	–	–
Cd	WA	–	–
	SE	0.49	−0,34
	GL	−0,20	0.46
	SL	−0,37	−0,43
	MU	–	–
	HE	–	–

(NRRT) to *U. cordatus* and copper (in senescent leaves of *R. mangle*, muscle and hepatopancreas of *U. cordatus*) and lead contamination (in sediment) (Table 1).

Fig. 3 presents the probability estimates of geno-cytotoxic damage in uçá crab as a function of lead and mercury contamination in abiotic compartments (for metals showing a significant association with biomarkers). The estimates were seen only for Pb (in water and sediment) and Hg (in sediment). The slope estimates (r), as well as the 50% probabilities (CG_{50}, genotoxic concentration; and CC_{50}, cytotoxic concentration) were significant ($p < 0.05$). For lead the probability of damage in 50% of the samples was 0.171 µg/g (in water) and 6.49 µg/g (in sediment), while for mercury this value was 162.8 ng/g (in sediment).

4. Discussion

Previous studies proved that *U. cordatus* is a good model for metal assessment, acting as important bioindicators of mangrove areas, due to its susceptibility to metal bioaccumulation (Pinheiro et al., 2012; Ortega et al., 2016), to sublethal damage responses (Pinheiro et al., 2013) and genetic diversity changes (Banci et al., 2017). In the present study, a conspicuous dose-responses relationship among all metals and biomarkers were not expected because the bioavailability and speciation of these contaminants are unknown (Rainbow, 2007). However, evidence of these associations were found here and confirm that *U. cordatus* is an effective metal bioindicator.

Abiotic contamination (water and sediment) by metals was linked to the accumulation in the biotic compartments (leaves and crab). This was revealed by the Mantel test, confirming the ecotoxicological model that deals with processes of transference of chemical elements from the environment to the organisms (Luoma and Rainbow, 2008). In addition, the data also confirm that metal contamination in *U. cordatus* are better explained by the concentration of these pollutants in the leaves of *R. mangle* than in water or in the sediment sample. This is explained by the use of the leaves of the mangrove litter as the main food item for the crab (Christofoletti et al., 2013), determining the contribution of this food source as a primary route of contamination in *U. cordatus*. Obviously, it should be considered that this is related to the selected metals and the crab tissues used for the analysis reported in the present study, however other metals (eg. Zn and As) and / or body structures (eg. gills and carapace), could have different routes of accumulation (Luoma and Rainbow, 2008).

The choice of biomarkers to evaluate sublethal damage in *U. cordatus* was based on the ecological relevance of the evaluated responses, as suggested by Bonassi et al. (2000), Neri et al. (2003) and Svendsen et al. (2004). According to these authors, cytotoxicity and genotoxicity can lead to cell death, starting a cascade of events that promotes tissues, organs and individual damage and consequently affect the entire crab population and community. The use of biomarkers such as micronuclei and lysosomal integrity in different species inhabiting various areas across the region covered by the OSPAR Convention, reveal the importance of cyto and genotoxicity levels in ecosystem assessments. Lysosomal membrane stability has recently been adopted by UNEP as part of the first tier of techniques for assessing harmful impacts within the Mediterranean Pollution Programme (OSPAR, 2013). In this way, they are promising tools for the detection of adverse effects caused by pollutants (Amiard-Triquet et al., 2013b; Amiard-Triquet and Amiard, 2013a; Duarte et al., 2016). The ecological relevance of the biomarkers

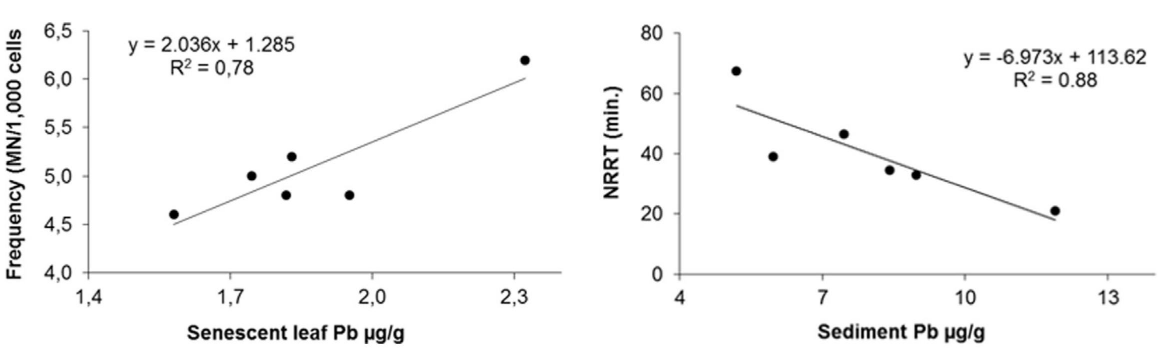

Fig. 2. Significant linear correlation ($p < 0.05$ and determination coefficients [R2] superior to 0.70) considering the empirical points of the relationship involving mean frequency of micronucleus (MN/1000 cells) and neutral red retention time (NRRT minutes) in *U. cordatus vs.* concentration of each metal (Cu, Cr, Mn, Hg, Pb and Cd) in the environmental compartments studied (water, sediment, leaves green/senescent of *R. mangle* and body structures of *U. cordatus*).

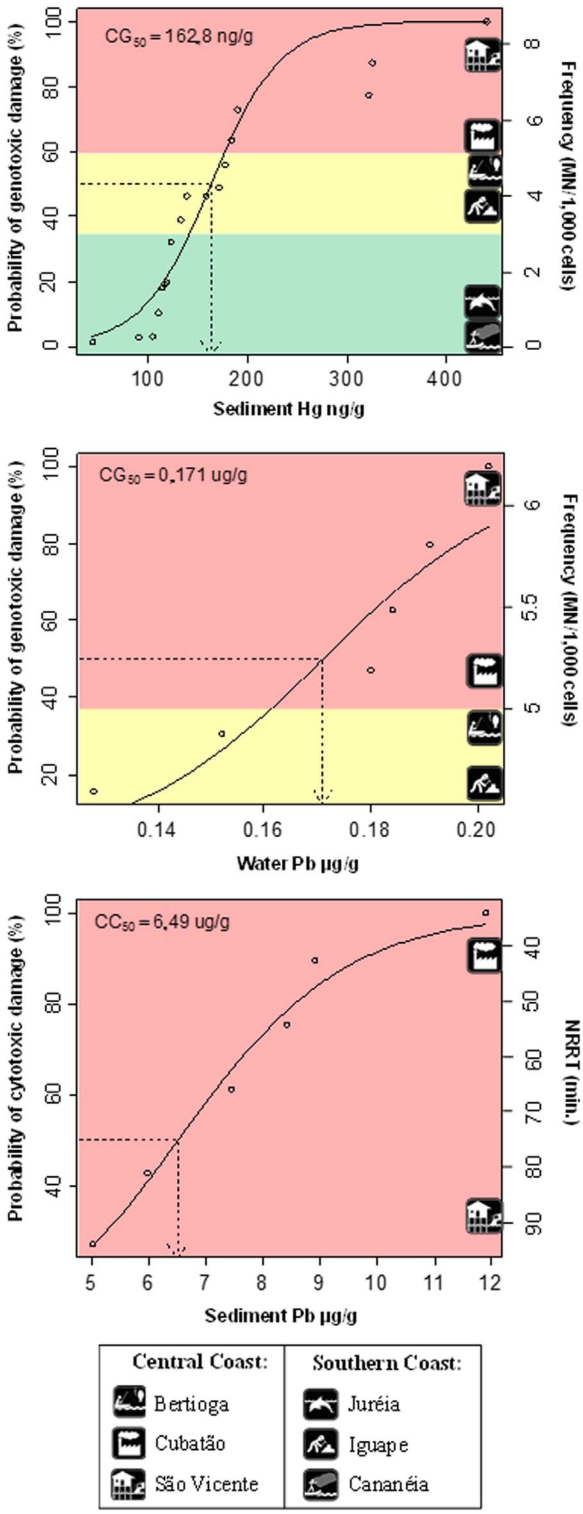

Fig. 3. Probability estimate of cytogenotoxic impact for *U. cordatus* as a function of Hg concentration (ng/g) in sediment, Pb (µg/g) in water and in sediment. Values obtained for micronucleus (MN‰) and neutral red retention time (NRRT) are also represented for each mangrove area using symbols. The black dashed arrows indicate the concentration where cytogenotoxic impact occurs at 50% probability (CG_{50}, genotoxic concentration; and CC_{50}, cytotoxic concentration). The colors within the plot area indicate the damage categories suggested for baseline values of genotoxicity (MN‰) and cytotoxicity (NRRT) in mangrove areas, according to Duarte et al. (2016), where: Green: PNI probability of no impact (PNI); Yellow: probability of low impact (PLI), and Red: probability of high impact (PHI). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

used in the present study indicate their use for assessing the conservation status of mangroves, allowing their proper categorization. This fact was confirmed through the response values of MN‰ (genotoxicity) and NRRT (cytotoxicity), in the following hierarchical sequences: SAV > (CUB = BET = IGU) > (CAN = JUR) and CUB > BET > SAV > IGU > CAN > JUR), respectively. Specifically for metal contamination, Pinheiro et al. (in preparation) diagnosed the environmental contamination of the same areas studied here as a function of metals in the abiotic compartments (water and sediment), verifying the following hierarchical sequence CUB > BET > (SAV = IGU) > CAN > JUR. Therefore, the results presented in the above study are consistent with a history of environmental contamination of these mangrove areas (Pinheiro et al., 2013, in preparation; Duarte et al., 2016; Banci et al., 2017), corroborating the data presented here.

Genetic damage can lead to genetic erosion and, above all, compromise the resilience of the species to selective environmental pressures (Bijlsma and Loeschcke, 2012). Cellular damage occurs by lipid peroxidation (LPO), when free radicals attack cell membranes, promoting structural and permeability changes, and resulting in cellular apoptosis. Thus, LPO is associated with aging and cancer, which may also be a response triggered by pollutants toxicity (Ferreira and Matsubara, 1997).

The neutral red assay is a biomarker endowed with broad plasticity for effectively demonstrating cytotoxic impacts due to contamination by organic or inorganic compounds (Svendsen et al., 2004). However, the possible relationship of genotoxicity and specific metal contamination is still questionable. Although this association is indicated in studies with different organisms and metal concentrations, whether it is lead (Çelik et al., 2005; Piao et al., 2007; Alghazal et al., 2008; Tapisso et al., 2009; García-Lestón et al., 2010), copper (Franke et al., 2006; Serment-Guerrero et al., 2011), chrome (Papageorgiou et al., 2008), cadmium (Seoane and Dulout, 2001; Bertin and Averbeck, 2006; Ahmed et al., 2010; Otomo and Reinecke, 2010), manganese (Gauthier et al., 2004; Erbe et al., 2011) and mercury (Al-Sabti and Metcalfe, 1995; Porto et al., 2005; Cavas, 2008). According to Luoma and Rainbow (2008), the hierarchy of toxicity by metals analyzed in the present study on aquatic organisms is as follows: Hg > Cu > Cd > Pb > Cr.

Positive and significant correlations were detected, involving the number of micronucleated cells (MN‰) *vs.* concentrations of Hg (sediment), Pb (water and green leaves) and Cu (hepatopancreas), as well as the negative significant correlations among mean neutral red retention time (NRRT) *vs.* concentrations of Cu (sediment and green/senescent leaves) and Pb (sediment). Although the correlation between two independent variables does not imply causality (Zar, 1999), these results argue in favor of a possible association between genomic damage, physiological integrity and the concentration increase of these metals in the above mentioned compartments. It is also noted that all compartments were associated with geno-cytotoxicity damage in the species studied here. Therefore, cytogenetic depletion may be related to contamination by several sources, requiring a more holistic view on pollution in estuarine systems.

Lead concentration in water samples, which were above the threshold limits established in Brazil (0,01 µg/g, according to Brasil, 2005), may be related to the observed genetic impact, as well as the concentration of this metal in the sediment (but below the guideline values – see PEL, Probable Effect Level; and TEL, Threshold Effect Level in Environmental Canada, 1999), leading to the highest physiological damage recorded. The same idea applies to the relationship between sublethal damage and the bioaccumulation of Pb and Cu in the leaves of *R. mangle* (without guidelines values established for plant contamination), as well as in relation to the concentration of Cu in the tissues of the crab. Although good mangrove quality is assured by Hg concentrations in the sediment below the PEL, the recorded contamination may also be determining the genotoxicity. Therefore, all these metals at low concentrations in the environmental compartments described above can determine the cytogenotoxic to the crab in the State of São Paulo mangroves.

It is important to note that genotoxicity tests are highly sensitive to genomic damage, even when pollutants are present at low concentrations (Monserrat et al., 2007). For example, experiments conducted by Sponchiado et al. (2011) detected genotoxic effects on the fish *Oreochromis niloticus*, even when submitted to low concentrations of 17β-estradiol (6 |g/L), after 24 h of exposure. In this scenario, it is believed that, although metals concentrations are relatively low, they may have genotoxic effect on the crabs in these studied areas. Similarly, the assessment of the integrity of the lysosomal membrane has a greater responsiveness in animals previously exposed to a greater variety and / or concentration of chemicals (Lowe et al., 1995; Svendsen et al., 2004). Therefore, both biomarkers were effective for indicating toxicity impact, being excellent methods for the differentiation between conserved and contaminated mangrove areas (Duarte et al., 2016). The present study also confirms *U. cordatus* as an excellent bioindicator of the conservation of mangroves status in view of their biological characteristics (Pinheiro et al., 2005, 2012, 2013; Nordhaus et al., 2009; Christofoletti et al., 2013) and considering the history of each studied area, as diagnosed by Pinheiro et al. (2012, in preparation) and Duarte et al. (2016).

However, it is important to emphasize that genetic damage and low physiological integrity can result from the synergistic effect of the metals studied here and other pollutants present in the mangrove areas, given the history of intrinsic contamination in each region (Gutberlet, 1996; Duarte et al., 2016; Pinheiro et al., in preparation). Other pollutants could include sulfur and nitrogen oxides, monoxide / carbon dioxide, methane, hydrocarbons, organochlorines, chlorinated phenols, fluorides, aldehydes, acids, particulate matter, all of which are already detected in the estuarine system of São Vicente and Cubatão (CETESB, 2001, 2007; Torres et al., 2015; Taniguchi et al., 2016), as well as emerging pollutants such as illicit drugs (Pereira et al., 2016). Therefore, it should be considered that other compounds not quantified in the present study could also influence the cytogenotoxic impacts measured here (Marchand et al., 2013).

A study previously conducted by Duarte et al. (2016), in the same mangrove areas of the present study, proposed guideline values to categorize the levels of cytotoxic impacts by NRRT (>120 min, “Probably No Impact”; 60–120 min, “Probable Low Impact”; and <60 min, “Probable High Impact”) and genotoxic by MN‰ (<3 MN‰, “Probably No Impact”; 3–5 MN‰, “Probable Low Impact”; and >5 MN‰, “Probable High Impact”). Although the bioavailability relevance of metals is recognized (Luoma and Rainbow, 2008), the probabilities of expected impacts greater than 70% reported here were associated with a “probable high impact “ for all cases, reinforcing the cytogenetic damages linked to the concentration of metals in the compartments.

Mercury, for example, has a genetic impact probability of 50% (CG_{50}) associated with 162.8 ng/g concentration in sediment, while the value of TEL for this metal (130 ng/g) was very close to the lower limit of the "probable low impact" category. Thus, the values of TEL and PEL for *U. cordatus* indicate an ideal criterion for the accumulation of Hg in mangroves. Thus, the results obtained in the present study ($CG_{50} = 162.8$ ng/g) corroborate with those found by Choueri et al. (2009). According to these authors, Hg concentrations between 80 and 320 ng/g are indicative of "moderate contamination", revealing "high contamination" when concentrations are above 320 ng/g.

Due to the presence of lead in the most contaminated areas (CUB and BET), it was not possible to observe the behavior of the probabilities in situations of "Probable No Impact" (Pb in water) and "Probable Low Impact" (Pb in sediment). In this case, the probabilities of 50% geno and cytotoxic impact were already in "probable high impact", in both water and sediment. The concentration obtained for such average probability was well above 0.01 μg/g, which is the reference concentration for quality brackish water (see CONAMA # 357/2005), although well below the values of TEL and PEL (30 and 110 mg/g in the sediment, respectively). The results obtained in the present study ($CC_{50} = 6{,}49$ μg/g) again align with the results found by Choueri et al. (2009), showing a "moderate contamination" when the concentration of Hg in the sediment ranged from 10.3 to 22.1 μg/g, with values indicating "high contamination" when above 320 ng/g. Therefore, it is perceived that the values established by the Canadian agency for lead were supposedly underestimated for *U. cordatus*, because with a concentration almost three times lower than the TEL, the species were already under a situation of “probable high” physiological impact. Obviously these results are correlations and projections, and it would be necessary to perform controlled laboratory experiments to test this premise. In addition, the sigmoidal model applied for probabilities determination has some limitations, since it assumes the highest sublethal damage observed as 100%, in addition to recommending a direct association (dose-response) between the involved variables (metal-biomarker). However, it should be taken into consideration that the Santos Estuary System has already been considered the most polluted estuary in the world (Silva et al., 2002). In addition, considering that the objective of this study was to evaluate correlations between variables that were statistically significant, it is believed that the probabilities estimates found are assertively reflecting the species exposure to the metals in the abiotic compartments.

The results obtained in the present study show the successful application of biomarkers to indicate areas under greater (or lesser) contamination by metals, and can be used as an additional tool in studies on conservation status of mangrove ecosystems. The effective use of *U. cordatus* as a sentinel species as well as the applicability of the two biomarkers in determining the conservation status of Western Atlantic mangroves has also been demonstrated, although other species of the same ecological value can be similarly studied in other mangroves around the world. Thus, studies with species of endemic brackish crustaceans would provide additional information on the conservation status of mangroves. Studies estimated that in 25 years (1980 and 2005) there was a subtraction of 20% (3.6 million ha) of mangroves areas, mainly for deforestation and other uses, the main ones being for shrimp farming (FAO, 2007). Unfortunately, Brazil follows this trend, and although mangrove conservation in Brazil is ensured by legislation (eg, Brazilian Forest Code, CONAMA Resolutions and the National Coastal Management Plan), several anthropic tensors have affected this ecosystem (Santos et al., 2014; Santos and Bitencourt, 2016), corresponding to a decrease in the Brazilian mangroves areas by more than 5%.

The information generated in this study can guide enforcement agencies to use effective measures for environmental management, based on a more integrative approach, using easier, faster and low operational cost protocols, as suggested here.

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