



## Mangrove metal pollution induces biological tolerance to Cd on a crab sentinel species subpopulation

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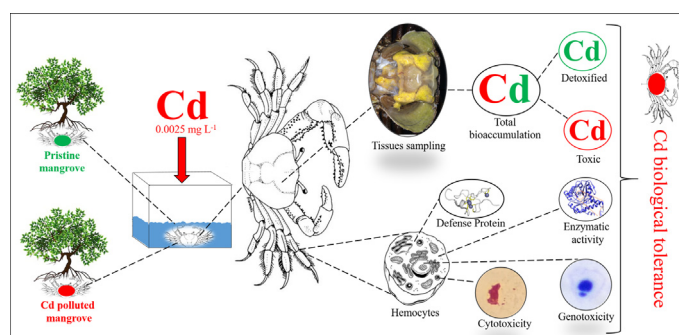
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### HIGHLIGHTS

- *Ucides cordatus* has a great capacity to immobilize cadmium in a non-toxic form.
- Bioaccumulation of cadmium is linked to geno-cytotoxic damages.
- Mangrove crabs from distinct areas presented different sublethal damages.
- Crabs from pristine mangroves accumulate more Cd in a toxic form in different tissues.
- Crabs from polluted mangrove are more tolerant to Cd.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Metals are persistent pollutants, able to accumulate in the biota and magnify in trophic web. In the specific case of cadmium contamination, it has been the subject of considerable interest in recent years because of its biological effects and it is one of major pollutant in estuarine areas. *Ucides cordatus* is considered a mangrove local sentinel crab species in Brazil and there are previous studies reporting crab subpopulations living from pristine to heavily metal impacted areas in São Paulo coast (Southeastern Brazil). Taking into account the background knowledge about these subpopulations, we proposed the hypothesis that crabs from a highly polluted mangrove (Cubatão - CUB) have developed biological tolerance to cadmium compared to animals from an Environmental Protected Area (Jureia - JUR). Aiming to verify this hypothesis, we have investigated total bioaccumulation and subcellular partition of Cd, besides biomarkers' responses during a long-term exposure bioassay (28 days, with weekly sampling) using a supposedly safe Cd concentration ( $0.0022 \text{ mg L}^{-1}$ ). Specimens from the pristine area (JUR) accumulated higher total Cd, as such as in its biologically active form in gills. Animals living in the polluted site (CUB) presented higher amounts of Cd in the mainly detoxifying tissue (hepatopancreas), which could be considered a pathway leading to tolerance for this metal. Multivariate analysis indicated that bioaccumulation (active, detoxified and total Cd) is linked to geno-cytotoxic damages. CUB subpopulation was considered more tolerant since it presented proportionally less damage and more capacity to allocate Cd in the main detoxifying forms and tissues.

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## 1. Introduction

Metals are pollutants of global concern because they may have large impact and present potential risk to the biota and are considered as important indicators of human local disturbance (Abraham and Susan, 2017; Duarte et al., 2017; Patel et al., 2018). Metal contamination becomes even more alarming due to current global warming (Cox et al., 2000), which increases concentrations of carbon dioxide in Earth's atmosphere and results in ocean acidification (Hoegh-Guldberg and Bruno, 2010). These changes will affect the distribution of different forms of trace metals making them more bioavailable and threatening (Chamizo et al., 2016; Stockdale et al., 2016).

However, biological populations may develop higher biological tolerance when exposed to metals for a long term (Zhao et al., 2015; Topal et al., 2017). This acquired resistance can originate as a result of physiological acclimation during the life cycle of the organism or from a population genetic adaptation by selective pressure over time (years, decades or even centuries, which culminate in mortality of more sensitive individuals) (Klerks and Weis, 1987; Terlizzi et al., 2005; Espinosa et al., 2007).

Among non-essential metals, cadmium (Cd) has become one of the focuses in aquatic toxicology, because it is one of the major pollutants in estuarine areas and it presents high toxicity and persistence. Anthropogenic terrestrial inputs are the main sources to estuaries areas, flowing into rivers and through mangrove ecosystems (Cresswell et al., 2017; Pedrosa et al., 2017). Cadmium tolerance has been observed in several crustaceans due to the development of biological strategies: *Tisbe holothuriae* (Bryan, 1994); *Gammarus pulex* (Stuhlbacher and Maltby, 1992); *Platynympha longicaudata* (Ross et al., 2002); *Eriocheir sinensis* (Silvestre et al., 2006); and *Chironomus riparius* (Pedrosa et al., 2017).

Biomarkers have received substantial efforts in assessing possible biological tolerances and they also have been applied in several environmental monitoring programs (Marine Strategy Framework Directive, 2008; Pereira et al., 2014), based on measuring sublethal biological disturbances (Pereira et al., 2011; Duarte et al., 2016, 2017). Thus, to estimate these damages, many authors have used techniques considered of large ecological relevance, e.g.: DNA strand breaks (DNA-SB) (Hartwig, 2018; Sarker et al., 2018); micronucleated cells (MN) (Hoshina et al., 2008; Pinheiro et al., 2013); neutral red retention time (NRRT) (Svendsen et al., 2004; Duarte et al., 2016, 2017); enzymatic activity of aminolevulinic acid dehydratase (ALA-D) (Alves Costa et al., 2007; Kalman et al., 2008); and metallothioneins (MTs) (Ahearn et al., 2004; Si and Lang, 2018). In addition, it is worth to note the importance of relating total metal bioaccumulation and subcellular partitioning (Campana et al., 2015; Cresswell et al., 2017) as supporting techniques to clarify some biological differences between subpopulations emerges.

Globally, there is a lack of knowledge about tropical ecotoxicology, especially in mangrove ecosystems. This habitat is considered a nursery for several animal species, among them, the “uçá”-crab (*Ucides cordatus*) is considered a local sentinel species by indicating metals pollution and determining the conservation status of mangrove areas (Pinheiro et al., 2013; Silva et al., 2018). At mangroves of southeastern Brazil, previous studies (Duarte et al., 2016, 2017) have shown first evidences that a crab subpopulation resident on a heavily impacted area (Cubatão Estuarine System) is supposedly more tolerant to metals compared to animals from Juréia-Itatins Ecological Station (JIES), an Environmental Protected Area.

This study aims to verify this hypothesis by identifying if the subpopulation of *U. cordatus* resident at Cubatão mangrove is more tolerant to cadmium exposure than animals from Juréia through bioaccumulation pathways, biological storage strategies and biomarker responses, considering water exposure route. It is also expected that this analysis would assist in elucidating Cd mode of action in *U. cordatus*. Finally, the study focuses on providing more evidence on Cd concentrations believed to be safe to avoid adverse biological effects by environmental legislation concerning water quality.

## 2. Material and methods

### 2.1. Field sampling at mangrove areas and collecting of *U. cordatus*

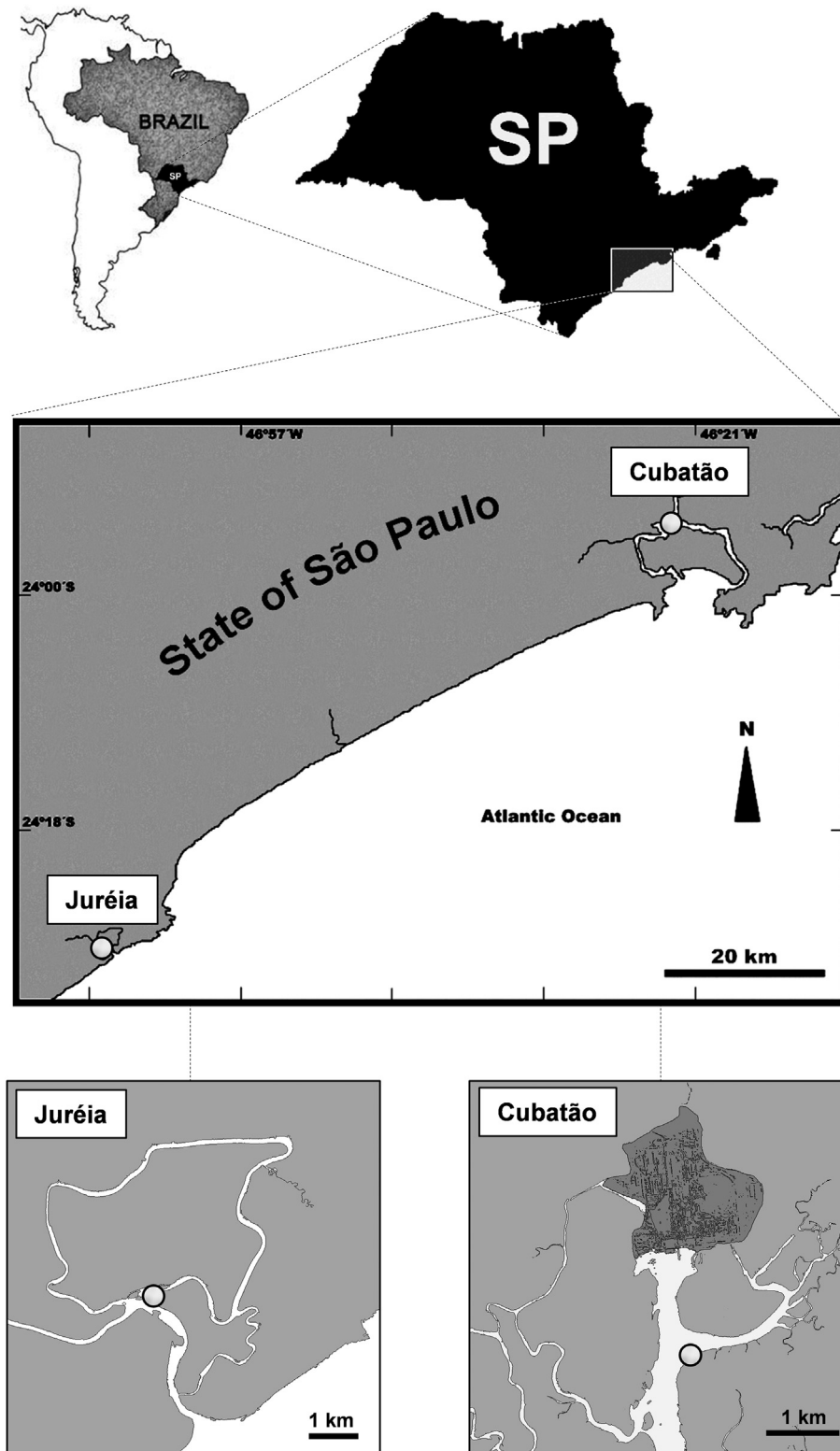
Crabs were collected in two mangrove areas located at the South-Central Coast of São Paulo State (Brazil) (Fig. 1), namely: **1) Juréia-Itatins Ecological Station, JUR** (24°26'03" S - 47°05'03" W): a non-polluted place located at a Conservation Unit. The human population is small and depends on traditional extractive activities that are free of threatening contaminants (Pinheiro et al., 2013; Duarte et al., 2016); and **2) Cubatão, CUB** (23°52'50" S - 46°22'25" W): considered one of the most polluted places in the world; in recent years, it has become the most important Brazilian industrial hub, formed by 23 industrial complexes, 111 factories, and >300 pollution sources. Home to the largest port in Latin America, it also experiences transit of large vessels carrying several types of chemicals (Catharino et al., 2008; Martins et al., 2011; Pinheiro et al., 2012, 2013; Duarte et al., 2016). The choice of these areas of study was based on the study conducted by Duarte et al. (2017) which identified Cd pollution at Cubatão mangrove and also confirmed Juréia mangrove as conservation area free from pollutants.

In both mangrove areas, only intermolt adult males (carapace width > 60 mm, avoiding any effect of sex, molting and life stage, as previously reported for other decapod crustaceans (Chou et al., 2000; Chen et al., 2005) were collected, specifically in a location with approximately 15 salinity (confirmed by an Atago S/Mill-E optical refractometer). Specimens were identified following the diagnostic characters of Melo (1996) and measured with precision calipers (0.01 mm). One hundred and sixteen specimens were hand-caught inside their galleries (76 animals from JUR and 40 from CUB) (Pinheiro et al., 2012). Animals were kept at 18 aquariums (50 cm long, 35 cm wide, 40 cm high and 4 mm thick) each of them containing 10 l of the control water (reference) (seawater free of contamination from outside ocean + distilled water) for fourteen days to acclimatization and depuration at a laboratory from the Federal University of São Paulo (UNIFESP/Brazil) (Reddy et al., 2011).

### 2.2. Ex-situ experiment of cadmium exposure, tissue sampling for bioaccumulation and biomarker analyses

After the acclimatization and depuration period, the animals were exposed to water dissolved Cd for 28 days (kinetic long-term exposure bioassay, Wang, 2011). The experiment consisted of three treatments: **1) Control (CONT)**, referring to animals from Juréia mangroves exposed to control water; **2) Juréia (JUR)**, animals also from Juréia mangrove exposed to Cd concentration below of the quality threshold according to Conama 357/2005 (0.0025 mg L<sup>-1</sup>); and **3) Cubatão (CUB)**, animals from Cubatão mangrove, also exposed to the same Cd concentration. Each treatment counted on five aquariums, each of them with 6 crabs, adding up 90 animals. Aquariums were cleaned, their water replaced, and their physico-chemical parameters monitored weekly (temperature, dissolved oxygen, conductivity, salinity, pH and reduction potential) as recommended by Artal et al. (2018). Animals were fed with *Rhizophora mangle* leaves from Juréia mangrove (30 g per crab per week, Christofolletti et al., 2013). Water aeration (24 h day<sup>-1</sup>) and photoperiod were constant (12 by 12 h: light-dark) for all experiment were adopted.

At every 7 days of exposition (T-0 (before the exposition to Cd); T-7; T-14; T-21 and T-28, where T is time in days) the defense mechanism to metals (by metallothionein content, MTs); the sublethal damages (genotoxic effect by strand breaks in DNA, DNA-SB; frequency of micronucleated cells, MN; and cytotoxic injury by neutral red retention time, NRRT); biochemistry disturbance by enzymatic activity of aminolevulinic acid dehydratase, ALA-D and the biological types of Cd storages (by subcellular partitioning, SCP) were monitored. DNA-SB quantification was performed in 84 hemolymph samples (3



**Fig. 1.** Locations of two mangrove areas (Juréia and Cubatão) where crabs (*Ucides cordatus*) were collected, at State of São Paulo, Brazil. (From Google Earth® satellite images by G. Pinheiro.)

specimens/treatment/exposition time) at laboratory of São Paulo State University (UNESP/Brazil) using the alkaline precipitation protocol detailed by Olive (1988). The quantity of DNA-SB is normalized and expressed using the total protein content ( $\mu\text{g}$  DNA/mg total protein TP). To MN and NRRT, 10 specimens/treatment/exposition time were analyzed, according to the methods described in detail in Duarte et al.

(2016). MN is expressed by the number of hemocytes that contained micronuclei to every 1000 cells analyzed (%) and NRRT is expressed by the retention time that the dye leak from lysosomes into the cytosol. MTs, ALA-D and SCP will be addressed in the next section.

A screening investigation was done to diagnose which two tissues accumulate Cd the most prior to SCP analyses. Twenty-five specimens

were sacrificed to dose the initial (T-0, n = 10 [CONT/JUR = 5 and CUB = 5]) and final (T-28 after the exposition: n = 15 [5 from each treatment]) concentrations of total Cd in five tissues: hemolymph, hepatopancreas, musculature, gills and carapace, totaling 125 samples. Samples were placed in plastic bottles, frozen and transported in ice gel to Nuclear and Energy Research Institute (IPEN – CNEN/SP), where Cd was determined. Samples were digested in a MARS 6 microwave oven using 65% (v/v) HNO<sub>3</sub> and Cd total quantification (detection limit = 1 µg/kg) was performed by ET AAS on a Perkin-Elmer Analyst 800 spectrometer (Athanasopoulos, 1994). NIST SRM 2976 (*Mussel Tissue*) was also analyzed in order to check the method validation. In addition, Cd was determined weekly in the aquarium treatment waters to confirm the exposure concentration (0.0025 mg L<sup>-1</sup>). The following QA/QC measures were adopted in this study.

### 2.2.1. Subcellular partitioning of cadmium (SPCd), metallothioneins (MTs), and activity of aminolevulinic acid dehydratase (ALA-D): sample preparation and quantifications

A total of 84 samples (3 crabs [replicas] × 2 tissues [hepatopancreas and gills, which accumulate Cd the most, see Results and discussion section] × 3 treatments [CONT, JUR and CUB] × 5 exposure times [T-0, T-7, T-14, T-21 and T-28]) were collected during the experiment of Cd exposition at UNIFESP/Brazil. They were stored in Eppendorf vials in a ultrafreezer (−80 °C) and, then, taken in dry ice to Institute of Marine Sciences of Andalusia (ICMAN-CSIC, Spain), where SCP, MTs and ALA-D studies were performed.

A Tris Buffer (applicable for the three analyses with pH adjusted to 8.2) was used to homogenize all tissues (ratio 1 [±0.25 g tissue]:4 [±1 mL Buffer]) using an Ultra-Turrax (IKAR T10 Basic) using a beaker with ice (4 °C). Homogenized tissues were subjected to differential centrifugation using temperature and digestion procedures, according to the protocol adapted from TAYLOR and MAHER (2012) and CAMPANA et al. (2015). Megafuge 16R (Thermo Scientific) and Optima Max-XP (Beckman-Coulter) centrifuges were used. Cd quantified in mitochondrias (P3), lysosomes and microsomes (P4), and on one enzyme pellets, and heat sensitive proteins (P5) were grouped as Biologically Active Metal (BAM) fractions, while Cd quantified in granule pellet (P2) and the final supernatant, containing heat stable metallothionein-like protein (S5) were grouped as Biologically Detoxified Metal fractions (BDM). Cd dosed in the supernatant (S2) fraction (nuclei and cellular debris) were not categorized in any of the cases (BAM neither BDM) (Taylor and Maher, 2012). ALA-D and total protein content (TP) analyses were performed using the S3 fraction. MTs were quantified using the S5 fraction (Taylor and Maher, 2012).

After the subcellular fractionation, a total of 504 cadmium quantifications were done in subcellular fractions by Inductively Coupled Plasma Mass Spectrometry (ThermoFisher ICAP-Q) (Taylor and Maher, 2012; Campana et al., 2015). For pellets fraction digestion, 1 mL of HNO<sub>3</sub> was added to each sample, which was next maintained for 1 h at 95 °C. Subsequently, all the digested samples from P2, P3, P4 and P5 were diluted in 10 mL of Milli-Q water for Cd quantification. Supernatant fractions, on the other hand, were diluted with 5% NaOH: 5 mL for S2 or 2 mL for S5 of Milli-Q water. Detection limit for SPCd was 0.009 ppb calculated as 3 \* SDs of blanks and LOQ 0.03 ppb calculated as 10 \* SD of blanks.

To ensure the quality assurance and quality control (QA/QC) of total bioaccumulation and subcellular partition of Cd some cautions were taken. A calibrated analytical balance and volumetric flasks (class A) were used. For total Cd concentration in the tissue, ET AAS was employed and the lamp sensibility was checked using control charts. For SPCd, the concentration, ICP-MS was employed and autotune and mass calibration were carried out periodically. Reference materials (NIST SRM 2976 and ERM CE-278k Sample 0066) were employed for checking the quality of results. No significant variation was found between measured and certified values. Blank and standard were run for each 10 samples.

ALA-D activity was performed according to the method of Nakagawa et al. (1995) and Hylland (2004). A total of 84 samples (3 crabs × 2 tissues × 3 treatment × 5 exposures times) from supernatants fractions of S3 (see Taylor and Maher, 2012) (hepatopancreas = 350 µL; gill = 150 µL) were analyzed. Calibration curves were conducted using aminolevulinic acid to porphobilinogen (PBG: 0, 1, 5, 10, 20 and 40 µL L<sup>-1</sup>) and blanks (0.1 mol L<sup>-1</sup> phosphate buffer, pH 6.2) were also used as reference. Readings were performed in a Microplate Reader (Infinite F200, TECAN) at 570 nm. Total proteins (TP) were also performed in all these same 84 samples, according to Bradford (1976). Activity of ALA-D enzyme is expressed as quantity of aminolevulinic acid to porphobilinogen (PBG) formed per hour from the Aminolevulinic Acid normalized with the total protein content (µg PBG/mg protein\*h).

MTs quantification was performed (same 84 samples) according methods adapted from Alhama et al. (2006, 2011) and Romero-Ruiz et al. (2008) by High-Performance Liquid Chromatography (HPLC), using S5 (see Taylor and Maher, 2012) (hepatopancreas = 32.2 µL supernatant + 32.2 µL homogenization buffer; gill = 64.4 µL supernatant [undiluted]). The quantity of MTs was also normalized and expressed using the total protein content (µg MT/mg total protein TP).

### 2.3. Statistical analysis and equations of metals concentration in the particulate fractions

Excel and R 3.5.1 were used to design the figures and perform statistical analyses (Ihaka and Gentleman, 1996). For exploratory data analysis, the independent variables (treatment and exposure time) were related to the dependent ones (SPCd, DNA-SB, MN, NRRT, MTs and ALA-D). Each of the obtained variables was previously submitted for a homogeneity of variances test (L, Levene's test) and normality (W, Shapiro-Wilk test). After the normal distribution confirmed (p > 0.05), the data set was submitted to an analysis of variance (ANOVA), comparing the averages by Tukey "a posteriori" test or Kruskal-Wallis (Zar, 1999). All analyzes were verified at 5% of statistical significance and performed them in R 3.5.1 (Ihaka and Gentleman, 1996). Net accumulation rates for BAM and BDM bioaccumulate Cd were calculated using the following equation (Campana et al., 2015):

$$\text{Daily net accumulation rate}_i = ([Cd]_t - [Cd]_{\text{control}}) / t$$

where i is either the bioaccumulated BAM or BDM cadmium; [Cd]<sub>t</sub> is the concentration of the specified cadmium (ng/g) measured at time t (days = 7); and [Cd]<sub>control</sub> is the same measured in the control organisms (CONT).

Multivariate analysis was applied to the complete data set to identify linkages between biological responses (SPCd and biomarkers) and independent variables (treatment and exposure time). Initially, the data were submitted to Chi-square and Hartley's tests to verify normality and homogeneity of variances, respectively. Thereafter, Factor Analysis, employing Principal Components Analysis (PCA) as the extraction procedure, analyzed the original data set. Data were rearranged in a correlation matrix and four factors were extracted considering eigenvalues higher than 1.0 (Kaiser's criteria). For the Factor Analysis, the variables were auto scaled (Varimax normalized) to be treated with equal importance. Variables having loadings >0.30 to a particular factor were considered associated to the respective factor (Tabachnic and Fidell, 1996). Besides, the analysis of the aggregated variables by PCA, the factor scores from each treatment/time exposure were employed in order to confirm the factor descriptions and also to characterize the independent variables. All analyses were performed using the PCA option of the multivariate exploratory technique procedure, followed by the basic setup for Factor Analysis procedure from R 3.5.1 (Ihaka and Gentleman, 1996).

### 3. Results and discussion

#### 3.1. Experiment validation and total bioaccumulation of cadmium

As alerted by ARTAL et al. (2018), it was prudently ensured that the main physical and chemical parameters were kept within the satisfactory levels to *U. cordatus* biology needs (Christofolletti et al., 2013; Pinheiro et al., 2018). They presented minor changes (temperature:  $21.5 \pm 0.5$  °C; dissolved oxygen:  $5.6 \pm 0.7$  mg L<sup>-1</sup>; conductivity:  $24843 \pm 476$  mS cm<sup>-1</sup>; salinity:  $15.8 \pm 0.6$  ppm; pH:  $7.6 \pm 0.1$ ; and redox potential:  $85.3 \pm 6.7$  mV). No significant differences between the treatments were observed according to Kruskal-Wallis ANOVA ( $p > 0.05$ ), thus ensuring no biased results.

Similarly, size and sex of the studied specimens were standardized. Selected crabs were adult males, the smallest animal (in carapace width, LC) was 60 mm long (126 g) and the largest was 91 mm long (329 g.). The overall mean width was  $76.0 \pm 7.6$  mm and mean weight was  $212 \pm 42$  g. There were no statistical differences between animal sizes and weights between the treatments, according to Kruskal-Wallis ANOVA ( $p > 0.05$ ).

Metal bioaccumulation and associated negative effects are complex phenomena, because they depend on several factors as uptake, distribution, storage and excretion (Gimbert et al., 2008; Bini et al., 2015). Biological effects of metals are associated to their specific chemical characteristics, speciation, kinetics and mainly environmental physico-chemical parameters (Rainbow, 2007). Cadmium is one of the most widespread aquatic toxic metal due to its expanded use in agriculture and industry (Pinot et al., 2000). This metal accumulates easily in organisms and causes several damage effects, including sublethal injuries (Ahmed et al., 2010; Bini et al., 2015). Zhu et al. (2018), for example, found histopathological changes and stress in expression response genes of the estuary mud crab *Scylla paramamosain*.

In the present study, crabs were exposed for a long term (28 days) to Cd concentration which assumes no environmental risks, according to Brazilian legislation (Conama 357/, 2005). Cd nominal concentration ( $0.0025$  mg L<sup>-1</sup>) in treatment waters (Juréia and Cubatão) was similar to the measured concentration (mean:  $0.0022 \pm 0.0006$  mg L<sup>-1</sup>). It is important to mention that such value is environmentally relevant, since it is currently found in the estuaries of the State of São Paulo (Pinheiro et al., 2012, 2013; Duarte et al., 2017).

Fig. 2 shows Cd bioaccumulation results in crab tissues (gills, carapace, gonad, hemolymph, hepatopancreas and musculature) at T-0 and T-28 days of exposure. There were not statistical differences between Control/Juréia and Cubatão treatments for the six tissues before the exposure (ANOVA Tukey's test,  $p > 0.05$ ). The highest overall average Cd concentration in crabs from Control treatment was observed in hepatopancreas ( $7.08$  µg/kg). Gills and hepatopancreas were the main tissues where this metal was accumulated in the exposed crabs, reaching a maximum of  $443$  µg/kg (crabs from Juréia treatment) and  $135$  µg/kg (crabs from Cubatão treatment) after 28 days of exposure. Comparing exposure treatments at the end of the experiment, it was possible to observe that crabs from Juréia treatment accumulated significantly more in gills ( $261 \pm 87$  µg/kg) than Cubatão treatment ( $129 \pm 45$  µg/kg), while crabs from this last treatment stored statistically higher in hepatopancreas ( $69 \pm 35$  µg/kg) than crabs from treatment ( $20 \pm 6$  µg/kg) (ANOVA Tukey's test,  $p < 0.05$ ). There was no statistical difference between these two treatments for the means measured in the carapace and muscle (ANOVA Tukey's test,  $p > 0.05$ ). No Cd detected accumulation was recorded in crabs from Juréia treatment after the exposure in hemolymph and gonads.

After the exposure period, the highest amount of Cd was recorded in gills for both treatments (JUR and CUB). This tissue is not usually the main one that accumulates this metal; Martín-Díaz et al. (2015) found a different pattern in the shore crab *Carcinus maenas*, which presented higher accumulation in hepatopancreas than in gills. The same was also observed for the crab *Charybdis longicollis*, the shrimp *Penaeus*

*semisulcatus* (Firat et al., 2007) and the blue crab *Callinectes sapidus* (Çoğun et al., 2017). All these species are found in the marine environment. However, estuarine species such as the mitten crab *Eriocheir sinensis* accumulated this metal preferentially in the gills (Cheng et al., 2018). This pattern was similar in *U. cordatus*, (Pinheiro et al., 2013; Ortega et al., 2016). This species is considered to present high hyper-regulatory capacities, obtaining an efficient osmoregulatory system and providing homeostatic equilibrium to the concentration of salts in its hemolymph (even in salinities varying between 2 and 33), which could alter the affinity and speed of the absorption and excretion of chemical substances (Leite and Zanotto, 2013). This may help explaining why estuarine species tend to accumulate more Cd in the gills (Ortega et al., 2016).

Differences were observed between crabs from conserved (JUR) and contaminated mangroves (CUB). Specimens from JUR accumulated higher Cd in the gills, while animals living in the polluted site presented proportionally higher quantity of this metal in the hepatopancreas, with both situations indicated statistically. Actually, crabs from CUB showed prominent bioaccumulations in almost all tissues (gonad, hemolymph and muscle). This fact could be related to that crabs from the conserved mangrove (JUR) will have relative physiological difficulties in allocating and storing this metal in other tissues or even eliminating it. Gills accumulated 87.5% of the metal for these animals. The results about daily net accumulation rates have confirmed such supposition, since Juréia treatment crabs have gills as the most vulnerable tissue for presenting higher daily active Cd uptake as long more time exposed. Crabs from CUB, however, were able to allocate a greater amount of this metal to mainly detoxifying tissue (hepatopancreas), which could be considered a form of adaptation and resistance to this metal (Rainbow, 2007; Eisler, 2010).

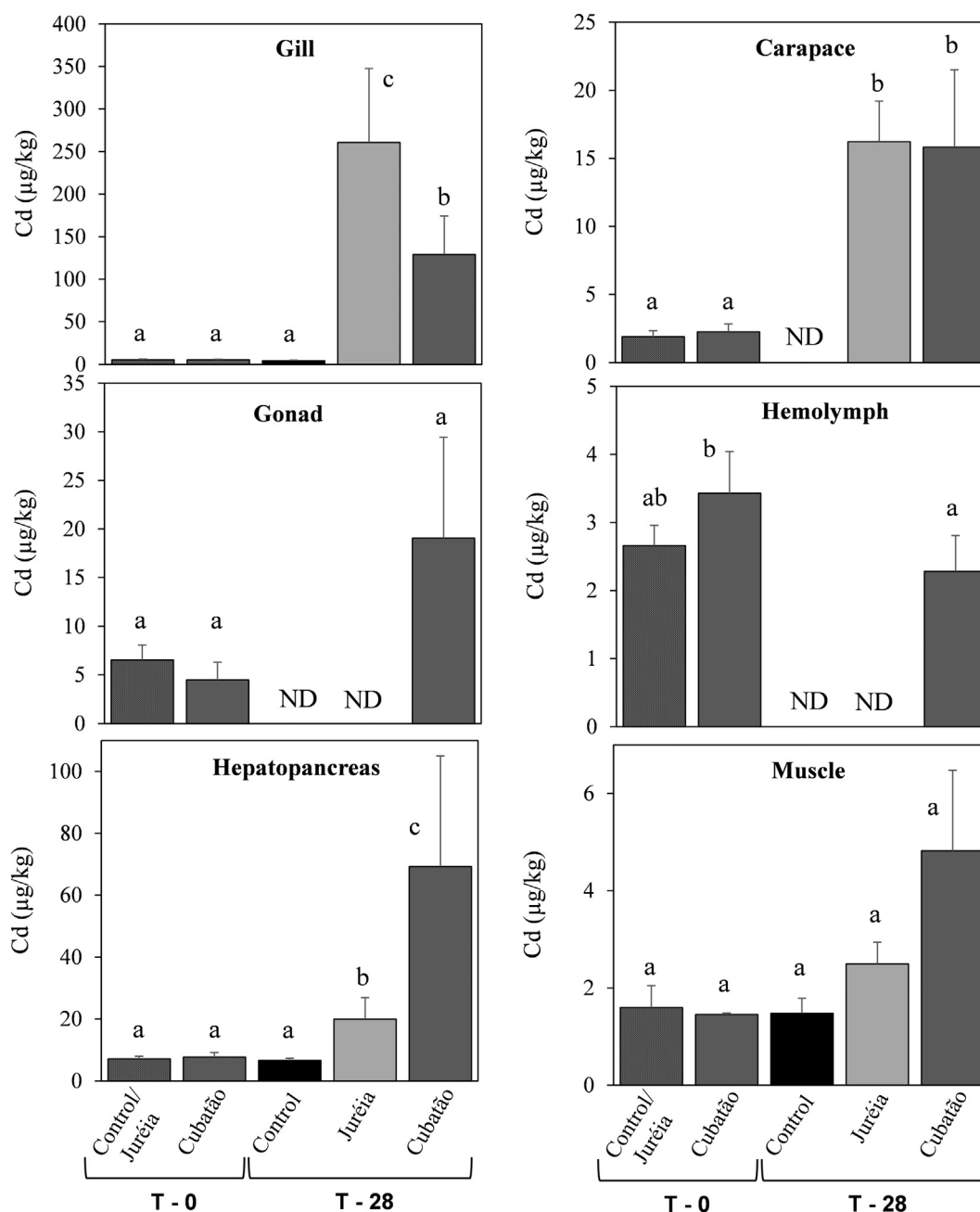
#### 3.2. Subcellular partition of cadmium

Cadmium can be stored in two forms, Biologically Detoxified Metal (BDM) and biologically available metal (BAM) (Campana et al., 2015). Subcellular partitioning of cadmium (SPCd) in hepatopancreas and gills are presented in Tables 1 and 2, respectively. A total of 504 quantifications of this metal were performed in subcellular fractions. These two tables present separately the results (mean and standard deviation) obtained for Biologically Active Metals (BAM = P3, P4 and P5) and Biologically Detoxified Metals (BDM = P2 and S5). After exposure to cadmium (T-28), this metal accumulated (ng/g) more expressively in BDM, namely Heat Stable – MT like proteins, and especially in the granules in both tissues.

During exposure to cadmium, the concentrations (ng/g) of BAM ranged from 0.02 (Jur T-14) to 358 (Cub T-28) for hepatopancreas and 0.006 (Jur T-7) to 1144 (Jur T-28) for gills. In terms of biologically detoxified, the concentrations (ng/g) of cadmium ranged from 113 (CONT T-14) to 1761 (CUB T-7) for hepatopancreas and 9 (CONT T-28) to 5561 (JUR T-28) to gills. The highest s of BAM (184 ng/g) and BDM (1602 ng/g) were observed in gills of crabs of Juréia treatment. Similarly, the lowest means of BAM (0.5 ng/g) and BDM (540 ng/g) were recorded in hepatopancreas of the same treatment. In addition, regardless of the treatment, BAM and BDM were highest in gills.

Comparing the data set between the treatments, biologically active and detoxified cadmium recorded in gills do not show significant differences between Juréia and Cubatão treatments (ANOVA, Tukey Test:  $p > 0.05$ ), however, biologically active cadmium observed in hepatopancreas of Cubatão treatment was statistically higher than Jureia treatment (ANOVA, Tukey Test:  $p < 0.05$ ) (Fig. 3).

Table 3 presents the relative proportion (percentages [%]) recorded to BDM and BAM in hepatopancreas and gills by treatment. During the exposure, BAM stood out in crabs from Cubatão after 28 days for both tissues (hepatopancreas [11%] and gills [16%]). However, the results observed to all treatments showed a large amount of metals in the particulate fractions considered biologically detoxified (always higher than



**Fig. 2.** Bioaccumulation of cadmium (concentrations in  $\mu\text{g}/\text{kg}$  and detection limit is  $1 \mu\text{g}/\text{kg}$ ) in six tissues of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) before (T-0) and after (T-28) Cd exposure at a concentration of  $0.0022 \pm 0.00006 \text{ mg L}^{-1}$ . Control and Juréia treatments belonged to the same group (Control/Juréia, dashed bars) and they were then divided after the seventh day (T-7). Details can be found in the methodology. Bars indicate the average, ND means “not detected”, vertical lines indicate the standard deviation, and different letters above the bars reflect statistical differences between averages according to Analysis of Variance (ANOVA) and by *a posteriori* multiple comparison with Tukey’s test ( $p < 0.05$ ).

80% in almost all cases). *U. cordatus* species stored Cd in detoxified form predominantly. This is considered a high amount since it has already been found about 45% in hepatopancreas and 60% in gills of *Crassostrea gigas* (Cao et al., 2018) and <40% to fiddler crab *Uca pugnax* (Houry et al., 2008). To other metals, for example Pb, Taylor and Maher (2012) found 66% detoxified in gills and 49% in hepatopancreas of the bivalve mollusk *Anadara trapezia*. Specifically, about the subcellular fraction of *U. cordatus* in the present study, cadmium was observed more in granules and heat stable metallothionein-like proteins (both gills and hepatopancreas). This result is in agreement with the literature (Taylor and Maher, 2012; Rosabal et al., 2015). Campana et al. (2015), for example, recorded a similar metal bioaccumulation strategy on bivalves where the vast majority of accumulated Cu is detoxified in granules form. The author attributed this fact probably resulting from

lysosomal breakdown of metallothionein-bound Cu as mentioned by Rainbow (2007).

Results on Cd daily net accumulation rates (BAM and BDM in  $\text{ng g}^{-1} \text{ tissue day}^{-1}$ , Table 4) showed that in Juréia treatment crabs, the gills are the most vulnerable tissue, since, the highest Cd daily active uptake rate (BAM) was reported. After 28 days of exposure, a Cd mean of  $66.35 \text{ ng/g/day}$  was accumulated in this tissue. While animals from Cubatão treatment demonstrated a similar pattern observed in the same period for hepatopancreas tissue, with a rate of  $17.20 \text{ ng/g/day}$  accumulated Cd. In relation to Cd stored in nontoxic form (BDM), Juréia treatment animals demonstrated a tendency to decrease intaking of this metal in hepatopancreas ( $5 \text{ ng/g/day}$  after 28 days) and to increase in gills (reaching  $413 \text{ ng/g/day}$  at the same period). Cd daily absorption mean values in tissues of Cubatão treatment crabs oscillated during the

**Table 1**  
Cd concentration (ng/g) accumulated in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM], mean  $\pm$  SD) from the hepatopancreas of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time (0 [before the experiment], 7, 14, 21 and 28 days) exposed to cadmium concentration ( $0.0022 \pm 0.00006 \text{ mg L}^{-1}$ ). The particulate fraction S2 does not belong to BAM neither BDM and ND means "not detected". Detection limit was 0.009 ppb calculated as  $3 * \text{SDs of blanks}$  and LOQ 0.03 ppb calculated as  $10 * \text{SD of blanks}$ .

Subcellular fractions		Exposure time (days)				
		T-0	T-7	T-14	T-21	T-28
CONT	Nuclei and cellular debris (S2)	11 $\pm$ 3	16 $\pm$ 6	193 $\pm$ 18	230 $\pm$ 13	10 $\pm$ 10
JUR			27 $\pm$ 10	503 $\pm$ 121	273 $\pm$ 372	275 $\pm$ 76
CUB		20 $\pm$ 5	72 $\pm$ 12	4657 $\pm$ 791	121 $\pm$ 78	295 $\pm$ 72
Biologically Active Metals (ng/g)						
CONT	Mitochondria (P3)	ND	ND	ND	ND	ND
	Lysosomes and microsomes (P4)	ND	1 $\pm$ 1	ND	0.2 $\pm$ 0.2	ND
	Enzymes (P5)	ND	0.8 $\pm$ 1	ND	0.05 $\pm$ 0.08	0.5 $\pm$ 0.7
JUR	Mitochondria (P3)		ND	ND	ND	ND
	Lysosomes and microsomes (P4)		0.2 $\pm$ 0.3	0.7 $\pm$ 0.7	0.3 $\pm$ 0.6	0.5 $\pm$ 0.7
	Enzymes (P5)		ND	ND	ND	0.4 $\pm$ 0.8
CUB	Mitochondria (P3)	ND	ND	14 $\pm$ 24	30 $\pm$ 52	98 $\pm$ 170
	Lysosomes and microsomes (P4)	0.08 $\pm$ 0.1	0.2 $\pm$ 0.2	12 $\pm$ 8	6 $\pm$ 4	7 $\pm$ 11
	Enzymes (P5)	15 $\pm$ 24	3 $\pm$ 2	14 $\pm$ 24	ND	15 $\pm$ 25
Biologically Detoxified Metals (ng/g)						
CONT	Granules (P2)	166 $\pm$ 19	211 $\pm$ 13	117 $\pm$ 9	114 $\pm$ 15	214 $\pm$ 52
	Heat stable - MT Like proteins (S5)	24 $\pm$ 10	201 $\pm$ 230	128 $\pm$ 142	27 $\pm$ 18	60 $\pm$ 44
JUR	Granules (P2)	166 $\pm$ 19	385 $\pm$ 89	335 $\pm$ 76	387 $\pm$ 116	270 $\pm$ 7
	Heat stable - MT Like proteins (S5)	24 $\pm$ 10	335 $\pm$ 242	344 $\pm$ 376	66 $\pm$ 26	43 $\pm$ 19
CUB	Granules (P2)	205 $\pm$ 24	353 $\pm$ 34	306 $\pm$ 38	356 $\pm$ 84	608 $\pm$ 361
	Heat stable - MT Like proteins (S5)	28 $\pm$ 12	653 $\pm$ 798	79 $\pm$ 9	107 $\pm$ 78	85 $\pm$ 42

exposition, reaching an uptake of 85 ng/g/day in hepatopancreas after 7 days and 273 ng/g/day in gills after 21 days of exposition. It is conspicuous there is a difference regarding the most vulnerable tissue for crabs from conserved (gill) and impacted (hepatopancreas) mangroves. For the latter mentioned animals, part of the Cd uptaken by their gills is translocated to other tissue, mainly to the hepatopancreas, which is considered the main storage and detoxifier tissue in crustaceans (Rainbow, 2007; Eisler, 2010). The same pathway was found to total bioaccumulation, as described before.

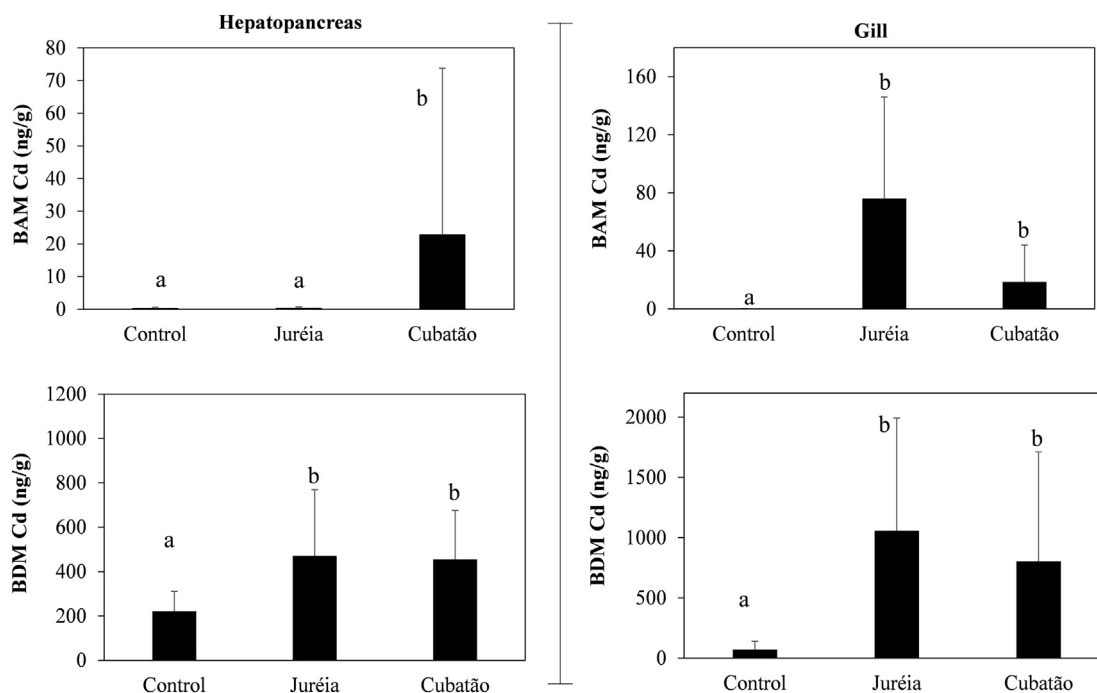
### 3.3. Sublethal damage

To thoroughly assess and indicate a supposed biological tolerance to cadmium by crabs from a polluted mangrove, a more holistic approach

must be performed (Silvestre et al., 2006; Pedrosa et al., 2017). Prediction of chemical resistance in a specific population is challenging because the susceptibility of aquatic organisms could intraspecific diverge due to differences in bioavailability, bioaccumulation kinetics, internal distribution and excretion of the metal, whose gathered, will determine or not the sublethal damages (Cresswell et al., 2017). Thus, biological disturbances are indicators of sensibility or threat to which crabs are subject (Pereira et al., 2014; Duarte et al., 2016, 2017). These adverse effects approaches ranging from down sublethal responses to up population-community level to of sentinel species are the current basis for most biomonitoring programs (Marine Strategy Framework Directive EU - MSFD, 2008/56/EC). Short-term responses on lower levels of biological organization have been used to detect first signs of impairment caused by xenobiotics (Pereira et al., 2014). Thus,

**Table 2**  
Cd concentration (ng/g) accumulated in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM], mean  $\pm$  SD) from the gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time (0 [before the experiment], 7, 14, 21 and 28 days) exposed to cadmium concentration in water ( $0.0022 \pm 0.00006 \text{ mg L}^{-1}$ ). The particulate fraction S2 does not belong to BAM neither BDM and ND means "not detected". Detection limit was 0.009 ppb calculated as  $3 * \text{SDs of blanks}$  and LOQ 0.03 ppb calculated as  $10 * \text{SD of blanks}$ .

Subcellular fractions		Exposure time (days)				
		T-0	T-7	T-14	T-21	T-28
CONT	Nuclei and cellular debris (S2)	ND	ND	ND	ND	ND
JUR			10 $\pm$ 6	94 $\pm$ 31	93 $\pm$ 117	119 $\pm$ 72
CUB		ND	3 $\pm$ 3	3 $\pm$ 4	54 $\pm$ 64	143 $\pm$ 93
Biologically Active Metals (ng/g)						
CONT	Mitochondria (P3)	ND	ND	ND	ND	ND
	Lysosomes and microsomes (P4)	0.01 $\pm$ 0.02	0.09 $\pm$ 0.15	0.1 $\pm$ 0.3	ND	0.1 $\pm$ 0.1
	Enzymes (P5)	0.02 $\pm$ 0.03	ND	ND	ND	0.2 $\pm$ 0.2
JUR	Mitochondria (P3)		ND	24 $\pm$ 24	238 $\pm$ 61	452 $\pm$ 589
	Lysosomes and microsomes (P4)		0.1 $\pm$ 0.1	2 $\pm$ 2	1 $\pm$ 2	4 $\pm$ 2
	Enzymes (P5)		0.6 $\pm$ 1	4 $\pm$ 2	0.9 $\pm$ 1	8 $\pm$ 4
CUB	Mitochondria (P3)	ND	ND	31 $\pm$ 39	19 $\pm$ 20	113 $\pm$ 131
	Lysosomes and microsomes (P4)	0.4 $\pm$ 0.6	0.7 $\pm$ 0.9	0.7 $\pm$ 0.6	0.2 $\pm$ 0.3	11 $\pm$ 12
	Enzymes (P5)	17 $\pm$ 21	0.1 $\pm$ 0.1	0.4 $\pm$ 0.9	0.4 $\pm$ 0.7	17 $\pm$ 22
Biologically Detoxified Metals (ng/g)						
CONT	Granules (P2)	16 $\pm$ 12	4 $\pm$ 6	1 $\pm$ 1	3 $\pm$ 5	9 $\pm$ 11
	Heat stable - MT Like proteins (S5)	309 $\pm$ 481	41 $\pm$ 37	123 $\pm$ 94	117 $\pm$ 70	17 $\pm$ 13
JUR	Granules (P2)	16 $\pm$ 12	88 $\pm$ 93	1257 $\pm$ 667	1127 $\pm$ 994	2619 $\pm$ 2417
	Heat stable - MT Like proteins (S5)	309 $\pm$ 481	60 $\pm$ 26	537 $\pm$ 124	414 $\pm$ 176	304 $\pm$ 191
CUB	Granules (P2)	0.1 $\pm$ 0.2	417 $\pm$ 667	43 $\pm$ 61	817 $\pm$ 888	428 $\pm$ 261
	Heat stable - MT Like proteins (S5)	51 $\pm$ 30	498 $\pm$ 187	326 $\pm$ 301	1217 $\pm$ 461	220 $\pm$ 121



**Fig. 3.** Concentration (ng/g) of cadmium accumulated in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM], mean  $\pm$  SD) from the hepatopancreas and gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) exposed to Cd concentration ( $0.0022 \pm 0.00006$  mg L<sup>-1</sup>). Different letters associated with the bars represent statistical differences between the treatment by ANOVA and Tukey's test ( $p < 0.05$ ). Detection limit for SPCd was 0.009 ppb calculated as  $3 \times$  SDs of blanks and LOQ 0.03 ppb calculated as  $10 \times$  SD of blanks.

biomarkers are considered pre-pathological indicators by having the capacity to detect biological dysfunctions in response to a stressor before population problems occur (Duarte et al., 2016). Changes in these biological indicators are conspicuous in organisms which inhabit impacted areas (Pereira et al., 2014). Table 5 presents the results about sublethal damages assessment using levels of enzymatic activity aminolevulinic acid dehydratase (ALA-D), Metallothioneins (MTs) content in hepatopancreas and gills, genotoxicity (DNA-SB and MN) and cytotoxicity (NRRT).

The ALA-D enzyme is considered essential to the metabolism, catalyzing the condensation of two molecules of aminolevulinic acid to porphobilinogen (PBG), which is an important phase for heme biosynthesis (function of oxygen bind in blood cells) (Kalman et al., 2008; Kayaalti et al., 2016). ALA-D enzymatic activity is usually inhibited by exposure to lead (Alves Costa et al., 2007) although there is incentive

in the literature to assessment of other metals, such as cadmium (Kalman et al., 2008) and mercury (Alves Costa et al., 2007). The protocol presented in this study was adapted and developed specifically to *U. cordatus*. During exposure to Cd, the levels of ALA-D ( $\mu$ g PBG/mg protein $\cdot$ h) ranged from 0.004 (CONT-0) to 1.85 (CUB T-14) for gills and ( $0.31 \pm 0.40$ ) and 0.46 (CONT T-0) to 5.40 (CUB T-28) for hepatopancreas ( $0.98 \pm 0.99$ ). An increase of ALA-D activity after 7 days of exposure to Cd was reported in hepatopancreas tissue from Juréia crabs ( $p < 0.05$ ). They showed higher enzymatic activity ( $3.02 \pm 0.10$ ). In the gills, the levels of ALA-D were higher ( $p < 0.05$ ) after 14 days in crabs from Cubatão treatment ( $1.47 \pm 0.52$ ). Alves Costa et al. (2007), for example, found an activity (ng PBG/min/mg protein) in blood (7.54), liver (37.7) and kidneys (75.4) of the fish *Halobatrachus didactylus*. These authors also evidenced that the blood of the fish *Hoplias malabaricus* was affected not only by lead but by methylmercury as well. Kalman et al. (2008) found an ALA-D inhibition measured in the whole tissues of *Chamelea gallina* linked with lead contamination ( $0.5$  to  $2$   $\mu$ g g<sup>-1</sup>). However, the results recorded in this study prevents a reliable diagnose

**Table 3**

Percentage of Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM] of Cd from the hepatopancreas and gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time (0 [before the experiment], 7, 14, 21 and 28 days) exposed to cadmium concentration in water (average =  $0.0022 \pm 0.00006$  mg L<sup>-1</sup>).

Exposure time (days)	Treatment	Hepatopancreas		Gills	
		BAM (%)	BDM (%)	BAM (%)	BDM (%)
T-0	CONT/JUR	0	100	0.07	99.93
	CUB	0.04	99.96	0.99	99.01
T-7	CONT	0.34	99.66	0.09	99.91
	JUR	0.05	99.95	0.35	99.65
T-14	CUB	0.62	99.38	0.13	99.87
	CONT	0.00	100.00	0.38	99.62
T-21	JUR	0.20	99.80	2.20	97.80
	CUB	8.69	91.31	11.74	88.26
T-28	CONT	0.17	99.83	0	100
	JUR	0.08	99.92	14.50	85.50
T-28	CUB	9.12	90.88	0.77	99.23
	CONT	0.18	99.82	1.74	98.26
T-28	JUR	0.30	99.70	9.67	90.33
	CUB	10.48	89.52	15.98	84.02

**Table 4**

Net accumulation rates of cadmium per day (ng Cd g<sup>-1</sup> days<sup>-1</sup>) as Biologically Detoxified Metals [P2 and S5 = BDM] and Biologically Active Metals [P3, P4 and P5 = BAM] from the hepatopancreas (left) and gills (right) of *Ucides cordatus* by treatment (Juréia and Cubatão) and exposure time (0 [before the experiment], 7, 14, 21 and 28 days) exposed to cadmium concentration in water ( $0.0022 \pm 0.00006$  mg L<sup>-1</sup>).

Exposure time (days)	Treatment	Hepatopancreas		Gills	
		BAM <sub>rate</sub>	BDM <sub>rate</sub>	BAM <sub>rate</sub>	BDM <sub>rate</sub>
T-7	JUR	0	43	0.09	14
	CUB	0.2	84	0.1	124
T-14	JUR	0.1	61	4	238
	CUB	5	20	4	35
T-21	JUR	0.01	44	34	203
	CUB	8	45	3	273
T-28	JUR	0.06	5	66	413
	CUB	17	59	20	88

**Table 5**  
Mean and standard deviation of DNA strand break (DNA-SB  $\mu\text{g DNA/mg prot}$ ), micronucleus frequency (MN/1000 cells), neutral red retention time (NRRT min), levels of enzymatic activity of aminolevulinic acid dehydratase (ALA-D in  $\mu\text{g PBG/mg prot}\cdot\text{h}$ ) and metallothioneins (MTs) content in tissues (hemolymph, hepatopancreas and gill) of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time (0 [before the experiment], 7, 14, 21 and 28 days) to cadmium concentration in water ( $0.0022 \pm 0.00006 \text{ mg L}^{-1}$ ). Different letters associate each treatment represent statistical differences between them into to the specific exposure time separately by ANOVA and Tukey's test ( $p < 0.05$ ).

Time	Treatment	DNA-SB ( $\mu\text{g DNA/mg prot}$ )		MN (%)		NRRT (min)		ALA-D ( $\mu\text{g PBG/mg prot}\cdot\text{h}$ )		MTs (mg MT/mg prot)					
		Hemolymph		Hemolymph		Hemolymph		Hepatopancreas	Gill	Hepatopancreas	Gill				
T-0	CONT/JUR	7.6 $\pm$ 2.3	a	1.2 $\pm$ 1.3	a	76.5 $\pm$ 8.5	b	0.7 $\pm$ 0.7	a	0.1 $\pm$ 0.1	a	0.06 $\pm$ 0.02	a	0.05 $\pm$ 0.006	a
	CUB	14.6 $\pm$ 5.0	a	7.2 $\pm$ 1.3	b	40.5 $\pm$ 7.2	a	1.0 $\pm$ 0.3	a	0.1 $\pm$ 0.1	a	0.06 $\pm$ 0.03	a	0.03 $\pm$ 0.01	a
T-7	CONT	10.9 $\pm$ 3.4	a	0.8 $\pm$ 0.8	a	73.5 $\pm$ 8.5	b	0.8 $\pm$ 0.2	a	0.1 $\pm$ 0.07	a	0.04 $\pm$ 0.003	a	0.03 $\pm$ 0.006	a
	JUR	12.3 $\pm$ 2.2	a	17.3 $\pm$ 3.8	c	50.0 $\pm$ 18.4	a	3.0 $\pm$ 0.1	b	0.2 $\pm$ 0.08	a	0.07 $\pm$ 0.02	b	0.05 $\pm$ 0.003	b
	CUB	14.7 $\pm$ 2.1	a	7.0 $\pm$ 1.6	b	40.0 $\pm$ 13.0	a	0.5 $\pm$ 0.2	a	0.08 $\pm$ 0.08	a	0.043 $\pm$ 0.004	a	0.04 $\pm$ 0.01	ab
T-14	CONT	8.6 $\pm$ 1.7	a	0.6 $\pm$ 0.9	a	70.5 $\pm$ 7.2	c	0.9 $\pm$ 0.2	a	0.5 $\pm$ 0.5	a	0.03 $\pm$ 0.005	a	0.04 $\pm$ 0.02	a
	JUR	14.1 $\pm$ 3.4	a	10.0 $\pm$ 1.4	b	51.7 $\pm$ 13.2	b	0.6 $\pm$ 0.2	a	0.1 $\pm$ 0.1	a	0.04 $\pm$ 0.01	a	0.05 $\pm$ 0.01	a
	CUB	12.6 $\pm$ 4.4	a	7.8 $\pm$ 1.6	b	39.0 $\pm$ 7.7	a	1.2 $\pm$ 1.3	a	1.5 $\pm$ 0.5	b	0.05 $\pm$ 0.02	a	0.05 $\pm$ 0.004	a
T-21	CONT	7.8 $\pm$ 2.5	a	1.4 $\pm$ 1.1	a	72.0 $\pm$ 6.7	b	1.0 $\pm$ 0.2	a	0.5 $\pm$ 0.4	a	0.04 $\pm$ 0.009	a	0.07 $\pm$ 0.02	a
	JUR	15.8 $\pm$ 1.4	a	8.5 $\pm$ 1.8	b	42.0 $\pm$ 6.7	a	1.2 $\pm$ 0.6	a	0.3 $\pm$ 0.4	a	0.05 $\pm$ 0.009	a	0.06 $\pm$ 0.01	a
	CUB	9.4 $\pm$ 1.2	a	7.2 $\pm$ 1.6	b	37.5 $\pm$ 8.7	a	0.6 $\pm$ 0.6	a	0.7 $\pm$ 0.9	a	0.04 $\pm$ 0.004	a	0.09 $\pm$ 0.02	a
T-28	CONT	10.9 $\pm$ 2.5	a	1.2 $\pm$ 1.3	a	75.0 $\pm$ 10.6	b	0.4 $\pm$ 0.08	a	0.2 $\pm$ 0.09	a	0.04 $\pm$ 0.002	a	0.04 $\pm$ 0.007	a
	JUR	20.4 $\pm$ 0.5	b	7.0 $\pm$ 0.8	b	39.0 $\pm$ 8.2	a	0.4 $\pm$ 0.2	a	0.2 $\pm$ 0.1	a	0.04 $\pm$ 0.01	a	0.04 $\pm$ 0.01	a
	CUB	7.2 $\pm$ 2.7	a	11.0 $\pm$ 1.0	b	35.0 $\pm$ 8.7	a	1.9 $\pm$ 3.0	a	0.1 $\pm$ 0.03	a	0.05 $\pm$ 0.03	a	0.04 $\pm$ 0.007	a

about Cd capacity to inhibit ALA-D activity, regardless of the mangrove of origin of the crab.

Metallothioneins (MTs) are low molecular weight proteins, considered the most important invertebrate defense agents to immobilize metals (Viarengo et al., 1999; Monserrat et al., 2007), especially in crustaceans (Ahearn et al., 2004). Environmentally relevant concentrations of metals induce MTs production through a genomic stimulation and their cytoplasmic presence maintains toxic metals chelation (Ahearn et al., 2004). During Cd exposure, MTs content (mg MT/mg prot) in exposed crab tissues ranged from 0.02 (CONT T-14) to 0.10 (CUB T-21) for gills ( $0.04 \pm 0.02$ ) and 0.02 (JUR T-28) to 0.09 (CUB T-28) for hepatopancreas ( $0.04 \pm 0.01$ ). There were no significant differences between the dosages observed in hepatopancreas in relation to the treatments ( $p > 0.05$ ), the exception, however, was observed after seven days of exposure to Juréia crabs, which metallothioneins content ( $0.06 \pm 0.01$ ) was statistically higher from the others two groups ( $p < 0.05$ ). To gills, also it was recorded only a higher quantity of metallothioneins on Juréia crabs if compared to Control and Cubatão treatments after seven days of exposure ( $p < 0.05$ ). Accordingly, crabs supposedly triggered their defense mechanisms producing more metallothioneins just after a few days of Cd exposure. The high dosage of these proteins was not maintained throughout the exposure period, perhaps a higher concentration of cadmium or some synergism with another metal in water could conceive the prolongation or higher values of MTs content. For example, Martín-Díaz et al. (2006) found that the combination of Cd ( $3 \mu\text{g L}^{-1}$ ) + Zn ( $700 \mu\text{g L}^{-1}$ ), produced the most significant metallothionein induction than isolated metals ( $1.01 \pm 0.56 \mu\text{g mg}^{-1}$ ). However, Ortega et al. (2016) found that the levels of metallothionein concentration for the same species (*U. cordatus*) were higher in a reference area (same mangrove of the present study) in hepatopancreas and gills and two seasons (rainy or dry) compared to other more contaminated areas. Although these results are not the expected pattern, similar results were also reported to the species *Procambarus clarkii* (Martín-Díaz et al., 2006) and *Carcinus maenas* (Legras et al., 2000). However, it's important to consider that these authors determined the metallothionein by differential pulse polarography, a method which has been shown by Pedersen et al. (2008) to present overestimated and wrong concentration determinations. In the present study, high levels of metallothionein concentration was also found in some cases in crabs from Control treatment, maybe due to laboratory conditions which provide oxidative stress (Ruttikay-Nedecky et al., 2013) and not recorded higher values necessarily in crabs exposed to cadmium. We agree, thus, with the hypothesis described by Ortega et al. (2016) about crabs from polluted areas not producing metallothionein proportionally (dose-response)

to metals concentration. The authors consider the physiological modifications of this species when coming from polluted areas and under constant pressure, allowing its survival without an unnecessary increase in the synthesis of metallothioneins, which would cause elevation of physiological stress, which, in theory, would not occur in crabs from mangroves not impacted by metals. In addition, Engel and Brouwer (1993), suggest that natural fluctuations of metallothionein levels in crustaceans may naturally occur, mainly due to their participation in the Cu/Zn-MTs regulatory system in each molting phase, decreasing in the pre-ecdise. In few words, just like the ALA-D enzyme, the MTs were also not biomarkers indicated for the elucidation of biological tolerance. This study presents specific protocols developed to *U. cordatus* species and could be performed in other future environmental studies.

Svensen et al. (2004) alert that both kinds of sublethal injuries can lead to cell death, directing a cascade impact (down to up), causing pathology in tissues, organs, death to individual and, consequently, determining in populations (Duarte et al., 2016) and communities disturbances (Pereira et al., 2011, 2014). Genomic damage, for example, can determine to genetic erosion and compromise the resilience of the species to the selective pressures of the environment (Monserrat et al., 2007; Bijlsma and Loeschke, 2012). Cd does not interact directly with DNA (Hartwig, 2018), it damages cell membranes by chronic exposure, because organisms develop defense mechanisms and increase of lipid oxidative stress, compromising in that way membrane permeability (Ortega et al., 2016, 2017) and promoting oxidative DNA damage (Dally and Hartwig, 1997; Hartwig, 2018). Other studies have also used genotoxicity as an ecotoxicological tool, for example, Sponchiado et al. (2011) detected genotoxic effects on *Oreochromis niloticus* fish, even when submitted to low concentrations of  $17\beta$ -estradiol (6 ng/L) after 24 h exposure. Ismail et al. (2018) showed a gradual increase in the frequency of micronuclei in *Labeo rohita* fish after exposure to an organophosphate pesticide for 96 h. Fossi et al. (2000) also evidenced a genotoxic impact in the crab *Carcinus aestuarii*, when contaminated by polycyclic aromatic hydrocarbons (PAHs). Sarker et al. (2018) found DNA damage in the marine rock oyster, *Saccostrea cucullata*, exposed to environmentally available metals along the Arabian Sea coast. During Cd exposure, the genotoxicity indicated by DNA-SB ( $\mu\text{g DNA/mg prot}$ ), demonstrated that crabs from control group presented 4.40 to 14.80 ( $9.17 \pm 4.0$ ), only differing statistically from Juréia ( $15.71 \pm 4.86$ ;  $p < 0.05$ ). Only in the last exposure time (T-28), Juréia crabs presented higher genotoxic damages ( $20.47 \mu\text{g DNA/mg prot}$ ) than Cubatão group ( $7.17 \mu\text{g DNA/mg prot}$ ). About the genotoxicity indicated by micronuclei, animals belonging to the control group presented 0 to 3 cells (MN%), with a mean of  $1.04 (\pm 1.05 \text{ MN}\%)$ , differing statistically

from the other two treatments in all exposure conditions ( $p < 0.05$ ). Specimens from Juréia treatment presented the highest variation of genotoxic damages observed (from 0 to 20 MN% cells, mean of  $8 \pm 5.45$  MN%). Animals from Cubatão presented always above 6 micronucleated cells per 1000 analyzed throughout the experiment (including before the exposure to the metal [T-0]). Only in the first week of exposure (T-7), the mean values of micronucleated cells between these two treatments differed significantly ( $p < 0.05$ ), registering higher mean values in the crabs from Juréia ( $17.33 \pm 3.78$  MN%) and lower in the animals from Cubatão treatment ( $7 \pm 1.58$  MN%).

Cytotoxic damages usually occur by lipid peroxidation (lipoperoxidation) due to free radicals reaction in the cell membranes, resulting in structural and permeability changes, leading in cellular apoptosis, also causing the same cascade effect at the higher levels of biological organization (Svendsen et al., 2004; Duarte et al., 2016). Pereira et al. (2014) found a relationship between cytotoxicity and ecological indexes, reinforcing the ecological relevance of this biomarker. During exposure to cadmium, crabs from Control group presented a neutral red retention time of  $73.5 (\pm 8.18)$  min and differed statistically from animals from Juréia ( $47.14 \pm 13.97$  min) and Cubatão treatments ( $39.16 \pm 8.98$  min) during the entire experiment. Only one statistical difference ( $p < 0.05$ ) was recorded in the second week of exposure (T-14) between crabs from Juréia ( $51.66 \pm 13.22$ ) and Cubatão treatments ( $39 \pm 7.74$ ). In this case, animals from Cubatão presented higher sublethal damages after two weeks of exposition.

#### 3.4. Evidences concerning about biological tolerance

The application of Multivariate analysis indicated that the original set of variables could be narrowed down to four new factors, those explained 83.05% of the total variance (Table 6). The first principal factor (F1) accounted for 33.5% of the variance and highlighted the gills as bioaccumulation tissue, since it presented relationship between DNA-SB, MN, NRRT, BAM, BDM and Cd total. The second principal factor (F2) accounted for 24.3% of the variance and pointed the hepatopancreas as bioaccumulation tissue, also presenting relationship between MN, NRRT, BAM, BDM and Cd total. The third principal factor (F3) accounted for 14.6% of the variance and presenting relationship between the biomarkers MN, ALA-D (in H) and MTs (in H). A negative association was recorded only in the last principal factor (F4). This factor accounted for 10.4% of the variance and the genotoxicity (DNA-SB) presenting negative relationship between MTs and BDM (both in gills). These findings reveal that the bioaccumulation of cadmium (active, detoxified and total) is linked to geno-cytotoxic damages, with difference in more vulnerable tissue: gills in crabs from conserved and hepatopancreas in

crabs from impacted mangroves. In addition, another interesting association was found between the bioaccumulated cadmium in the gills, the metallothionein production and DNA-SB damage consequently. The multivariate investigation is evidencing an attempt of crabs from JUR in immobilizing the metal in this tissue, but without success. Therefore, geno-cytotoxic results recorded in present study were more evident in terms of biological sensitivity indicators than the last two biochemical biomarkers already discussed above.

The values recorded for the biomarkers in control group were statistically different from the crabs exposed to cadmium, but still it is essential to discern the relevance of such observed disturbances. Duarte et al. (2016) proposed guide values (PNI: probable null impact; PLI: probable low impact; and PHI: probable high impact) categorizing these genetic and physiological damages which the *U. cordatus* species could be submitted, based on the same biomarkers used in the present study. After exposure to cadmium, 100% of the crabs belonging to the Juréia treatment presented a “probable high genotoxic impact (PHGI)” and 64.28% of them showed a “probable high cytotoxic impact (PHCI). Whereas 95.65% animals from Cubatão group revealed PHGI and 94.44% of them a PHCI. In short, according to guide values of these authors, crabs from a conserved mangrove demonstrated a higher genetic sensitivity while animals from an impacted one have a higher physiological vulnerability for cadmium.

It is interesting to elucidate whether the resident crabs of an impacted mangrove by cadmium have developed a higher biological tolerance for this metal. First, it was found that crabs from JUR presented a higher genotoxic effect after the first week, a gradual and significant reduction of this injurie just after that, assuming that crabs were able to trigger their genetic repair mechanisms more efficiently (as also observed by Ortega et al., 2016), but without achieving the changes considered normal. Regarding cytotoxicity, a difference was recorded after fourteen days of exposition to cadmium, where crabs from a impacted mangrove presented relatively higher injurie, without differences observed temporarily, suggesting that crabs were already physiologically stressed even before of exposure to cadmium and without effect observed during the 28 days. While crabs from Juréia mangrove have the same cytotoxicity observed in crabs from impacted one after seven days and did not present any type of recovery, indicating that these animals were also more susceptible to the physiological effects of cadmium contamination. In general, all sublethal damages observed in crabs from polluted mangrove were low compared to the crabs from pristine mangrove during the Cd exposure. Therefore, perhaps Cubatão crabs do not have the same ordinary health quality as the animals living in a pristine area, even because they were exposed to a wide range of organic and inorganic pollutants during their whole life (Martins et al., 2011; Pinheiro et al., 2012, 2013; Duarte et al., 2016, 2017). However, it is expected that these animals have developed more tolerance to cadmium due to their capacity to allocate this metal to a detoxifying tissue (hepatopancreas).

**Table 6**

Sorted rotated factor loadings of the original variables on the four principal factors from Factor Analysis, employing by Principal Components Analysis as the extraction procedure. Four factors were extracted considering eigenvalues higher than 1.0 (Kaiser's criteria). Only variables having loadings  $>0.30$  to a particular factor were considered associated to the respective factor.

Variance (%)	Factor 1	Factor 2	Factor 3	Factor 4
	33.5	24.3	14.6	10.4
MN	0.32	0.62	0.65	
NRRT	0.57	0.54		
BAM (Cd in H)		0.30		
BDM (Cd in H)		0.98		
Cd total - H		0.97		
BAM (Cd in G)	0.95			
BDM (Cd in G)	0.88			0.31
Cd total - G	0.91			
ALA-D - H			0.94	
ALA-D - G				
MTs - H			0.65	
MTs - G				0.80
DNA SB	0.74			-0.31

#### 4. Conclusions

Resident subpopulations from a pristine mangrove showed relevant sublethal geno-cytotoxic damages by exposure to Cd concentrations at levels that can be considered safe by environmental regulations. Different patterns of accumulation were found in crabs from pristine and impacted areas. Crabs from JUR (pristine) showed higher concentration in gill tissue whereas for crabs from CUB (impacted), the hepatopancreas was the main Cd storage organ. CUB subpopulation was considered more tolerant because it presented proportionally less damage and more capacity to allocate Cd to detoxifying tissues. Crabs from JUR were more sensitive, even exposed at low Cd concentration. The information from this study provided new insights on Cd effects and safe concentrations, which could help to manage mangrove ecosystems according to differential conservation status.

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