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Lead toxicity on a sentinel species subpopulation inhabiting mangroves with different status conservation

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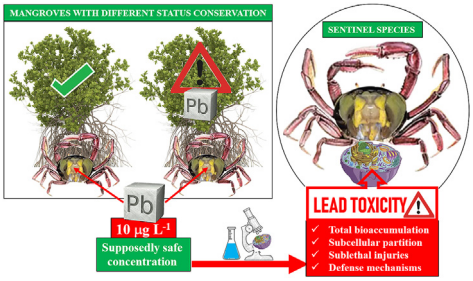
HIGHLIGHTS

- Sentinel species showed high ability to allocate lead in detoxified forms.
- Lead toxicity is observed even at concentrations considered environmentally safe.
- Defense mechanisms and cyto-genotoxic damage were recorded.
- Bioaccumulation (total, active and detoxified) is linked to biomarkers.
- Crabs from polluted mangrove may have developed biological tolerance to lead.

GRAPHICAL ABSTRACT



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ABSTRACT

Lead is a priority pollutant introduced in the aquatic environment by different sources commonly located in estuarine regions, such as ports, marinas and industries. Environmental agencies around the world set the maximum allowable concentration of lead in effluents, surface water and sediment, but few studies reported its accumulation and chronic toxicity in mangrove benthic invertebrates using concentrations believed to be safe. In the case of Brazilian mangrove environments, *Ucides cordatus* is a crab species of choice to be used in bioaccumulation studies. We have assessed biomarkers' responses (DNA strand breaks, micronucleated cells, metallothioneins, enzymatic activity of aminolevulinic acid dehydratase and neutral red retention time) and the total bioaccumulation in six tissues of *U. cordatus* crabs resident to mangrove areas under different conservation status during a 28-day period bioassay. We also investigated Pb subcellular partition and biomarkers' responses using a supposedly safe concentration (10 µg L^{-1}). During the Pb exposure, the highest concentration of Pb was observed in crab gills. Crabs also showed a high ability to allocate Pb in detoxified forms. Multivariate analysis pointed out that bioaccumulation (total, active and detoxified) is linked to biomarkers. Even in supposedly safe dosage, U. cordatus triggered its defense mechanisms expressing more metallothioneins and presented relevant

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cyto-genotoxic damage. Our data suggest the development of biological tolerance to Pb in crabs from polluted areas. Our results provided a new insight about lead toxicity even at concentrations considered environmentally safe, which could support new strategies to manage estuarine areas considering their respective conservation status.

1. Introduction

Among the contaminants released in water bodies, metals are among those of greater persistence and toxicity (Ahearn et al., 2004; Rainbow and Black, 2005; Rainbow, 2007; Abraham and Susan, 2017). Pollution by these contaminants has attracted worldwide attention, because of their high impact and potential risk, since they easily accumulate in the biota and may be transferred along the trophic chain (Ahearn et al., 2004; Rainbow, 2007; Vilhena et al., 2012; Trevizani et al., 2018). Metals accumulated in seafood can also produce exposure in humans and human health problems including cancer have been identified and related to metals (Järup, 2003; Fouani et al., 2017).

Estuarine systems and mangroves are located precisely in regions where human activities are frequent and intense. Besides, these ecosystems have physicochemical characteristics which contribute to metal immobilization, providing relevant biogeochemical barrier to them (Pinheiro et al., 2013; Duarte et al., 2017).

Kulkarni et al. (2018) reviewed the global distribution of metals in mangrove forests (abiotic and biotic compartments) and highlighted that lead (Pb) is one of the most studied, widespread and toxic metal. The reason is due to its particular properties (softness, malleability, durability, low melting point, and resistance to corrosion), which makes Pb to be used in several human activities, especially in industries (Flora et al., 2012). Lead, as a non-essential metal, does not have any biological function. It is known to cause severe and irreversible damage to local biota (Costa et al., 2018; Meng et al., 2018). Church et al. (2017) have updated species sensitivity distribution evaluations for acute (100 μg L^{-1}) and chronic (10 μg L^{-1}) Pb toxicity to saltwater aquatic life, however there is a lack of studies in tropical regions.

In estuarine systems, several studies have shown environmental concerns about the threats to crustacean species (Eisler, 2010; Verslycke et al., 2003; Ferrer et al., 2006; Costa et al., 2018). Crabs, for example, are supposedly more vulnerable to lead than fish, since they usually have geographically more restricted locomotion ability and may bioaccumulate proportionally more metals (Das et al. 2007). Specifically, about these organisms, several authors have indicated the lead toxicity to them, for example, Krishnaja et al. (1987) indicated histopathological effects of this metal to *Scylla serrata*. Also, during lead exposure, glutathione peroxidase activity and lipid peroxidation increased in *Parasesarma erythodactyla* were observed (Macfarlane et al., 2006). Ferrer et al. (2006) pointed out acute effect to larvae of *Chasmagnathus granulate*. Choueri et al. (2009) also documented Pb toxicity leading to alterations in benthic macrofauna. Pan et al. (2011) observed genotoxic effects in gills and hepatopancreas of *Charybdis japonica* even at low concentrations. Li et al. (2016) observed its harmful effects on several reproductive endpoints of *Sinopotamon henanense* and Li et al. (2017) evidenced the toxic effects of Pb by entering through Ca^{2+} channels of the same species (Leite and Zanotto, 2013). Duarte et al. (2017) found a significant association between the accumulation of this metal (in water, sediment and green leaves of *Rhizophora mangle*) with physiological and genetic damages recorded in *U. cordatus*. Xu et al. (2019) observed that *Charybdis japonica* presented immunological suppression, and enhanced levels of metallothioneins and HSP70 gene expression induced by lead.

Biomarkers have the capacity to assess such sublethal injuries (Monserrat et al. 2007; Pereira et al., 2011, 2014; Pinheiro et al., 2013; Duarte et al., 2016, 2017) and they also have been evaluated in several environmental monitoring programs (Silva et al., 2018). DNA strand breaks (DNA-SB) (Hartwig, 2018; Sarker et al., 2018); micronucleated cells (MN) (Hoshina et al., 2008; Gusso-Choueri et al., 2016; Duarte et al., 2016, 2017); metallothioneins (METALO) (Ahearn et al., 2004; Si and Lang, 2018); enzymatic activity of aminolevulinic acid dehydratase (ALA-D) (Alves Costa et al., 2007; Kalman et al., 2008; Fernández et al., 2015); and neutral red retention time (NRRT) (Svendsen et al., 2004; Pereira et al., 2014) are among the biomarker assessment techniques considered of strong ecological relevance (Amiard-Triquet et al., 2013). Furthermore, the association of biomarker responses to total metal bioaccumulation and subcellular biopartioning is important in metal toxicity assessment (Campana et al., 2015; Cresswell et al., 2017; Duarte et al., 2019), being this last technique capable of elucidating metal handling by the organisms and identifying the potential concern in a risk assessment context (Urien et al., 2018).

Mangroves have distinct ecosystem functions (providing ecological, social and economic services) and there is a global lack of knowledge about tropical mangrove ecotoxicology. The *Ucides cordatus* crab is a species of choice in metal bioaccumulation studies in order to indicate the conservation status in mangroveforests (Pinheiro et al. 2013; Duarte et al., 2017; Silva et al. 2018). However, some species may acquire higher biological resistance after long term metal exposure (Espinosa et al., 2007; Zhao et al., 2015; Topal et al., 2017). Duarte et al. (2019) have demonstrated that an *U. cordatus* subpopulation from a polluted mangrove was considered more tolerant to Cd than a preserved one, since it presented proportionally less sublethal damage and more capacity to allocate the metal in the main detoxifying forms and tissues (see details about adaptation to metals in aquatic organisms in Klerks and Weis, 1987).

Few studies reported lead accumulation and its toxicity in benthic macro invertebrates. Then this study aims to assess these aspects on sentinel species subpopulations obtained in mangroves under distinct conservation status, identifying if such subpopulations present differences in their respective biological tolerances to Pb. For this objective, pathways for bioaccumulation, storage strategies and biomarker responses were investigated with the expectation of elucidating the mode of action of Pb in *U cordatus*. Finally, the study was aimed at providing extra evidence on the Pb levels for water quality legislations in order to be considered safe to avoid adverse biological effects at marine environments.

2. Material and methods

2.1. Sampling of U. cordatus crabs

Crabs were sampled in April 2017 under official license to collect zoological material provided by the Brazilian Institute of the Environment and Renewable Natural Resources (SISBIO/IBAMA-

MMA) to LFAD (Grant # 24845-1), as well as the authorization from the Research Ethics Committee of Federal University of São Paulo (UNIFESP/HSP) (Process# 2511010714). Two mangrove areas were targeted for the sampling of the *U. cordatus* specimens. Both of them are located at the southern coast to São Paulo State, Brazil. The first mangrove site is located at a restricted protect area and is considered to be very pristine: **Juréia-Itatins Ecological Station**, **JUR** (24°26′03″ S - 47°05′03″ W). The second mangrove area, **Cubatão, CUB** (23°52′50″ S - 46°22′25″ W), was once considered one of the most polluted costal zones worldwide due to intense industrial discharges In order to avoid any sex, molting and life stage effects, only intermolt adult males (carapace width > 60 mm) were collected (Chou et al., 2000; Chen et al., 2005). Melo (1996) diagnostic characters were used for specimen identification using a 0.01 mm precision caliper. Eighty animals from JUR and 43 from CUB were hand-caught from their galleries and for acclimatization and depuration; they were kept for 14 days in 18 aquariums (50 cm long, 35 cm wide, 40 cm high and 4 mm thick) containing 10 L of control water each. The control water, used as reference, was adjusted with contamination-free seawater and distilled water, in order to obtain a salinity of 15.

2.2. Lead exposure experiment

Crabs were exposed to a Pb concentration of 10 μg L^{-1} for a 28-days period, after 15 days of acclimatization (Wang, 2011). This concentration was obtained dissolving P. A. $PbNO_3$ in water, according to procedures described by Duarte et al. (2019)., This concentration level is below the water quality threshold for Pb, according to CONAMA 357/2005 from the Brazilian Environmental Legislation (Brasil, 2005) but it is slightly higher than the North American (EPA, 2017 = 5.6 μg L^{-1}) and European Council (DIRECTIVE 2008/105/EC, 2008 = 7.2 μg L^{-1}) legislations. Three treatments were considered in this study: **1) Control (CONT),** referred to Juréia mangrove animals exposed only to control water; **2) Juréia (JUR)** and **3) Cubatão (CUB)**, respectively referred to crabs from Juréia and Cubatão mangroves, exposed to Pb at the 10 μg L^{-1} concentration. Detailed information on experimental design can be found in Supplementary Material.

2.2.1. Tissue sampling for the assessment of Pb accumulation and biomarker response

Twenty-five crab specimens were sacrificed to dose the initial and final concentration of total Pb in five tissues: hemolymph, hepatopancreas, musculature, gills and carapace, totaling 125 samples. For the initial concentration, 10 specimens were used (CONT/JUR = 5 and CUB = 5) while for the final 28-day exposure, 15 specimens were used (5 from each treatment).

For biomarker responses, at every 7 days of exposure (T-0; T-7; T-14; T-21; T-28), three specimens per treatment were sacrificed for the analysis of the following biomarkers: metallothioneins contents, METALO); DNA strand breaks in DNA, DNA-SB; frequency of micronucleated cells, MN; neutral red retention time, NRRT); enzymatic activity of aminolevulinic acid dehydratase, ALA-D) and the biological types of Pb storages (by subcellular partitioning, SPPb).

DNA-SB quantification was performed in 84 hemolymph samples using the alkaline precipitation protocol and is expressed as the total protein content (μg DNA/mg Total Protein, TP). (Olive, 1988). For MN and NRRT analyses, 10 specimens/treatment/exposure time were employed, according to the procedures found at Duarte et al. (2016). MN is expressed by the number of hemocytes that contained micronuclei to every 1000 cells analyzed (‰) while NRRT is expressed by the retention time that the neutral red dye leaks from lysosomes into the cytosol.

2.2.2. Sample preparation and quantifications for the subcellular partitioning of lead (SPPb), metallothioneins (METALO) and activity of aminolevulinic acid dehydratase (ALA-D)

For these quantifications, 84 samples (3 specimens [replicates] x 2 tissues [hepatopancreas and gills],] x 3 treatments [CONT, JUR and CUB] x 5 exposure times [T-0, T-7, T-14, T-21 and T-28]) were collected. Hepatopancreas and gills were chosen as these are the preferential organs for Pb accumulation as describes in the Results section. Samples were stored in Eppendorf vials in an ultra-freezer (−80 °C), until, according to the procedures described in Duarte et al. (2019) and Supplementary Material.

2.3. Statistical analysis and Pb fraction concentration equation

The R environment 3.5.1 (Ihaka and Gentleman, 1996) was used for the analyses and statistical significance was verified at 5% using the following packages: *GPArotation* (Bernaards and Jennrich, 2005), *FactoMineR* (Le et al., 2008; Husson et al., 2012), *Factoextra* (Kassambara and Mundt, 2017) and *Psych* (Revelle, 2018). For exploratory data analyses, the independent variables (treatment and exposure time) were related to the dependent ones (SPPb, DNA-SB, MN, NRRT, METALO and ALA-D). Before analysis, variables were previously submitted homogeneity of variances (L, Levene's test) and normality (W, Shapiro-Wilk test) tests. After confirming the normal distribution ($p > 0.05$), the data set was submitted to an analysis of variance (ANOVA), comparing the mean values by Tukey or Kruskal-Wallis tests (Zar, 1999).

For the estimation of the Pb BAM (biologically available metal) and BDM (biologically detoxified metal) daily net accumulation rates the following equation was employed (Campana et al., 2015):

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

where i is the bioaccumulated BAM or BDM lead; $[Pb_i]t$ is the concentration of the specified lead (ng g^{-1}) measured at time t (days = 7); and $[Pb_i]$control is the concentration of the specified lead (ng g^{-1}) measured in the control individuals (CONT).

To evaluate associations between biological responses (SPPb and biomarkers) and independent variables (treatment and exposure time), multivariate analysis was applied to the data set. Prior to the ANOVA and multivariate analyses, data normality and homogeneity of variances were submitted to Chi-square and Hartley's tests, respectively. Afterwards, Factor Analysis, employing Principal Components Analysis (PCA) as the extraction procedure, was used to analyze 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. High loading variables (>0.30) to a particular factor were considered associated to the respective factor, as proposed by Tabachnic and Fidell (1996). To confirm factor descriptions and also to characterize the independent variables in the analysis of the PCA aggregated variables, factor scores from each treatment/time exposure were employed.

3. Results

3.1. Experiment validation and total lead bioaccumulation

Minor changes were observed in the physical and chemical parameters during the experiment (temperature: 21.7 ± 0.4 °C; dissolved oxygen: 6.0 ± 0.6 mg L^{-1}; conductivity: 23918 ± 260 mS cm-1; salinity: 15.5 ± 0.1 ppm; pH: 7.6 ± 0.05; and redox potential:

102.3 ± 9.5 mV) and significant differences between the treatments were observed according to Kruskal-Wallis ANOVA ($p < 0.05$). During the 28-day experiment, a Pb concentration of 10.6 ± 1.4 μg L^{-1} was used for exposition of the animal. Detected Pb was statistically lower in the CONT treatment and before exposure (T-0) in almost all tissues, validating the experiment.

Selected crabs were adult males with carapace width (LC) varying between 61 mm long (92 g) to 90 mm long (234 g). Mean width and weight were 75.0 ± 5.6 mm and 144 ± 27 g, respectively. No statistical differences on animal size and weight were observed among treatments according to Kruskal-Wallis ANOVA ($p > 0.05$).

Results on Pb bioaccumulation in crab tissues (gills, carapace, gonad, hemolymph, hepatopancreas and musculature) at T-0 and T-28 days of exposure are presented in Fig. 1. There were no statistical differences between Control/Juréia and Cubatão treatments for the six tissues before the exposure (ANOVA Tukey's test, $p > 0.05$). The highest Pb concentration in Control treatment crabs was observed in gills (365 ± 144 ng g^{-1}). The preferential tissues for Pb

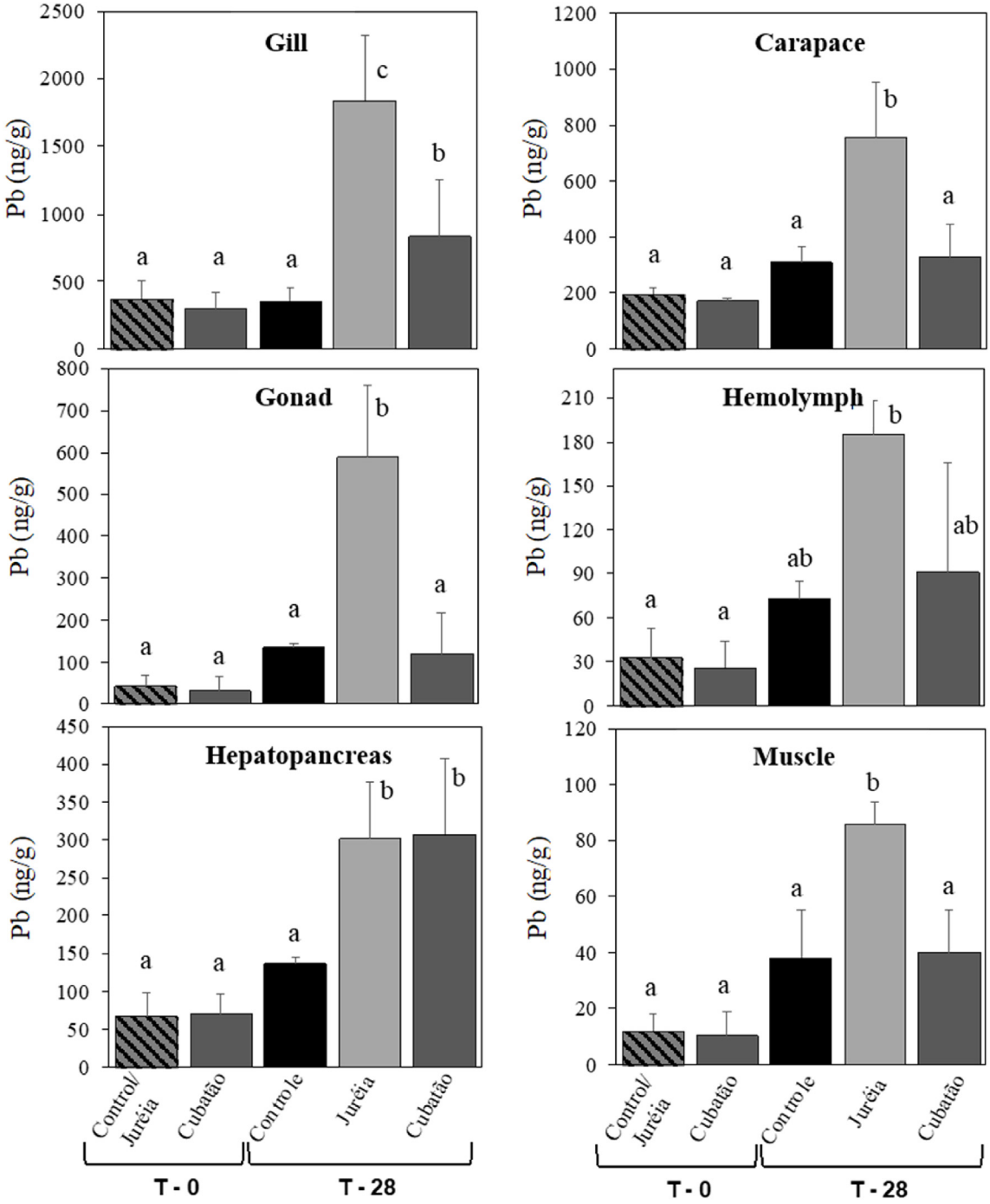

Fig. 1. Bioaccumulation of lead (concentrations in ng/g and detection limit is 1 ng g^{-1}) in six tissues of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) before (T-0) and after (T-28) Pb exposure at a mean concentration of 10.6 ± 1.4 μg L^{-1}. Control and Juréia treatments belonged 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, 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 ($p < 0.05$).

accumulation after the 28-day exposure were gills and carapace from Juréia treatment crabs (maximum of 3184 ng g^{-1} for gills and 1220 ng g^{-1} for carapace). At the end of the experiment, it was possible to observe that JUR crabs accumulated significantly more in gills (1835 ± 490 ng g^{-1}), carapace (757 ± 198 ng g^{-1}) and gonad (589 ± 171 ng g^{-1}) than animals from CUB. However, for hemolymph, hepatopancreas and muscle, no statistical difference between these two treatments was observed. (ANOVA Tukey's test, $p > 0.05$).

3.2. 3.2. Pb subcellular partition (SPPb)

According to Campana et al. (2015), Pb can be stored in two forms, biologically detoxified metal (BDM) and biologically available metal (BAM). Pb was determined a total of 504 subcellular fractions. Subcellular partitioning of lead (SPPb) in hepatopancreas and gills are presented in Tables 1 and 2, respectively; which show 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). Pb accumulated more expressively in BDM in heat stable-like proteins, and especially in the granules in both hepatopancreas and gills. For hepatopancreas, BAM mean Pb concentrations ranged from 1 ng g^{-1} (CONT) to 51 ng g^{-1} and for gills, it ranged from 1 ng g^{-1} (CONT) to 1009 ng g^{-1} (JUR T-28). In terms of BDM, mean Pb concentrations ranged from 156 ng g^{-1} (CONT T-28) to 1187 ng g^{-1} (CUB T-21) for hepatopancreas and from 134 ng g^{-1} (CONT T-28) to 6874 ng g^{-1} (JUR T-28) for gills. In addition, BAM and BDM were highest in gills, independent of the treatment. However, JUR and CUB treatments observed in gills did not present significant differences in BAM and BDM results as presented in Fig. 2 (ANOVA, Tukey Test: $p > 0.05$).

Table 3 presents the relative proportion recorded to BDM and BAM in hepatopancreas and gills by treatment. BAM stood out for CUB crabs after the 28-day exposure for hepatopancreas (10%) and gills (18%). However, all treatments presented large amounts of Pb (always > 95%) in the particulate fractions, which are considered biologically detoxified.

BAM and BDM Pb daily net accumulation rates (ng g-1 day-1), presented in Fig. 3, suggests that for JUR, the gills are the most vulnerable tissue, as the highest Pb daily active uptake rate (BAM) was reported. After 28 days of exposure, a Pb mean of 148 ng g-1 day-1 was accumulated in this tissue. While CUB animals presented a pattern similar to the observed in this tissue to BAM, with a rate of 42 ng g-1 day-1 after 28 days. In relation to Pb stored in nontoxic form (BDM) after the 28-day exposure, JUR animals presented a tendency to increase the Pb intake in hepatopancreas (163 ng g-1 day-1) and gills (781 ng g-1 day-1). Pb daily absorption mean values in tissues of CUB crabs oscillated during the exposure, reaching an uptake of 363 ng g-1 day-1 in hepatopancreas at T-14 and 473 ng g-1 day-1 in gills at T-7.

3.3. 3.3. Defense mechanisms and sublethal damage

Table 4 presents the sublethal response assessment using levels of enzymatic activity aminolevulinic acid dehydratase (ALA-D), Metallothioneins (METALO) content in hepatopancreas and gills, genotoxicity (DNA-SB and MN) and cytotoxicity (NRRT).

The mean levels of ALA-D (μg PBG/mg protein h) ranged from 0.371 (CUB T-14) to 1.964 (JUR T-21) for hepatopancreas and 0.046 (JUR T-21) to 3.67 (JUR T-7) for gills. CONT animals (1.796 ± 0.431) presented higher activity than JUR (0.665 ± 0.349) and CUB animals (0.623 ± 0.138) at T-7. Hepatopancreas from CUB crabs showed reduced activity of this enzyme at T-7 (0.62 ± 0.14) and T-14 (0.371 ± 0.096) ($p < 0.05$).

Metallothionein mean content (mg MT/mg prot) in exposed crab tissues ranged from 0.026 (CONT T-21) to 0.065 (CUB T-21) for

Table 1
Pb concentration (ng/g, mean ± SD) in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM]) from the hepatopancreas of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time T (0 [before the experiment], 7, 14, 21 and 28 days) exposed to lead concentration (10.6 ± 1.4 μg 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*SDs of blanks and LOQ 0.03 ppb calculated as 10*SD of blanks.

Subcellular fractions		Exposure time (days)														
		T-0			T-7			T-14			T-21			T-28		
Cont	Nuclei and cellular debris (S2)	125	±	72	116	±	2	51	±	2	55	±	48	155	±	142
Jur					212	±	80	67	±	14	182	±	49	182	±	107
Cub		122	±	15	229	±	73	164	±	63	236	±	149	288	±	200
Biologically Active Metals (ng/g)																
Cont	Mitochondria (P3)	ND			ND			ND			ND			ND		
	Lysosomes and microsomes (P4)	1	±	2	3	±	3	ND			5	±	6	6	±	2
	Enzymes (P5)	8	±	3	1	±	1	1	±	1	3	±	3	4	±	3
Jur	Mitochondria (P3)				ND			ND			ND			10	±	18
	Lysosomes and microsomes (P4)				4	±	6	5	±	4	5	±	2	3	±	3
	Enzymes (P5)				3	±	3	5	±	7	10	±	3	12	±	7
Cub	Mitochondria (P3)	ND			ND			7	±	11	ND			51	±	64
	Lysosomes and microsomes (P4)	4	±	1	ND			6	±	6	10	±	5	12	±	10
	Enzymes (P5)	5	±	5	6	±	7	10	±	7	10	±	4	5	±	5
Biologically Detoxified Metals (ng/g)																
Subcellular fractions		Exposure time (days)														
		T-0			T-7			T-14			T-21			T-28		
Cont	Granules (P2)	467	±	179	444	±	101	358	±	63	367	±	236	156	±	85
	Heat stable - MT Like proteins (S5)	653	±	731	166	±	94	283	±	106	782	±	932	759	±	947
Jur	Granules (P2)				872	±	56	911	±	258	623	±	219	1168	±	787
	Heat stable - MT Like proteins (S5)				259	±	42	324	±	288	725	±	580	888	±	1085
Cub	Granules (P2)	360	±	30	827	±	243	943	±	116	1187	±	383	581	±	238
	Heat stable - MT Like proteins (S5)	1141	±	1191	1110	±	969	2237	±	2430	2267	±	1328	265	±	144

Table 2
Pb concentration (ng g-1) accumulated in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM], mean ± SD) from the gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time T (0 [before the experiment], 7, 14, 21 and 28 days) exposed to lead concentration in water (10.6 ± 1.4 μg 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*SDs of blanks and LOQ 0.03 ppb calculated as 10*SD of blanks.

Subcellular fractions		Exposure time (days)														
		T-0			T-7			T-14			T-21			T-28		
Cont	Nuclei and cellular debris (S2)	1	±	2	ND			ND			ND			ND		
Jur					14	±	23	1	±	3	9	±	8	64	±	40
Cub		ND			6	±	8	10	±	9	56	±	66	60	±	103
Biologically Active Metals (ng/g)																
Cont	Mitochondria (P3)	ND			13	±	23	ND			51	±	88	ND		
	Lysosomes and microsomes (P4)	6	±	6	1	±	1	2	±	2	6	±	5	6	±	3
	Enzymes (P5)	3	±	3	3	±	3	2	±	3	4	±	3	2	±	2
Jur	Mitochondria (P3)				ND			8	±	14	125	±	128	1009	±	747
	Lysosomes and microsomes (P4)				2	±	2	6	±	2	3	±	2	17	±	7
	Enzymes (P5)				6	±	5	23	±	13	11	±	5	20	±	6
Cub	Mitochondria (P3)	ND			92	±	159	189	±	62	297	±	487	1003	±	1231
	Lysosomes and microsomes (P4)	5	±	3	6	±	4	6	±	0	10	±	8	8	±	4
	Enzymes (P5)	12	±	15	9	±	7	9	±	4	7	±	5	12	±	15
Biologically Detoxified Metals (ng/g)																
Subcellular fractions		Exposure time (days)														
		T-0			T-7			T-14			T-21			T-28		
Cont	Granules (P2)	199	±	64	283	±	125	154	±	73	934	±	257	134	±	44
	Heat stable - MT Like proteins (S5)	46	±	6	26	±	10	81	±	68	77	±	69	146	±	140
Jur	Granules (P2)				2160	±	843	3662	±	2299	2747	±	576	6874	±	1202
	Heat stable - MT Like proteins (S5)				179	±	197	39	±	38	66	±	17	80	±	43
Cub	Granules (P2)	1596	±	1255	3465	±	2636	2029	±	1662	3637	±	2940	3772	±	2185
	Heat stable - MT Like proteins (S5)	46	±	12	158	±	203	177	±	251	56	±	47	146	±	162

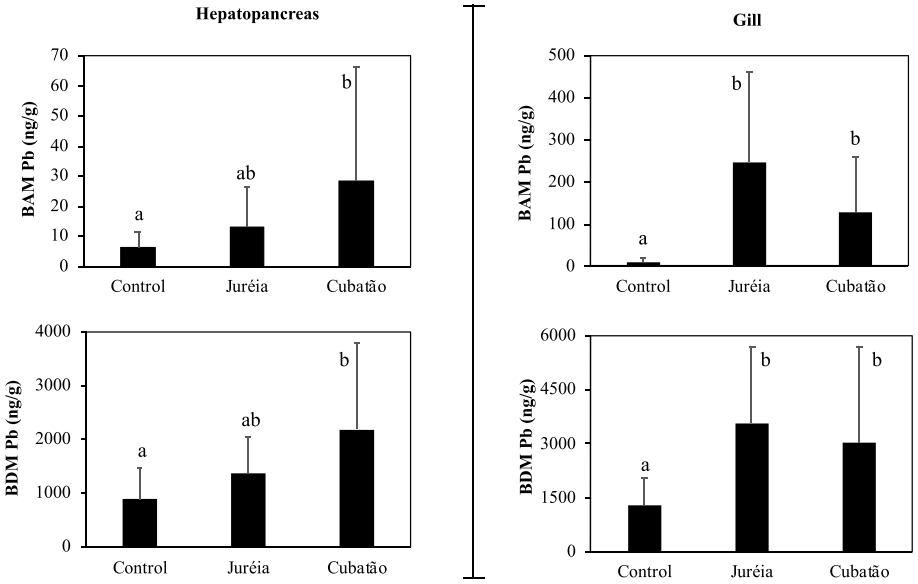

Fig. 2. Concentration (ng/g) of lead accumulated in the subcellular fractions (Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM], mean ± SD) from the hepatopancreas and gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) exposed to Pb concentration (10.6 ± 1.4 μg L^{-1}). Different letters associated with the bars represent statistical differences between the treatment by ANOVA and Tukey's test ($p < 0.05$). Detection limit for SPPb was 0.009 ppb calculated as 3*SDs of blanks and LOQ 0.03 ppb calculated as 10*SD of blanks.

gills and 0.023 (CONT T-14 and T-21) to 0.061 (CUB T-28) for hepatopancreas. There were no significant differences between the dosages observed in hepatopancreas in relation to the treatments ($p > 0.05$), the exception, however, was observed for CUB at T-28, which METALO content (0.061 ± 0.011) was statistically higher than the others two groups ($p < 0.05$). In gills, higher quantities of metallothioneins were observed at JUR and CUB comparing to CONT group at T-7 and T-21 ($p < 0.05$). CUB hepatopancreas presented significantly higher METALO contents at T-28 ($p < 0.05$). Concerning to gills, JUR crabs presented higher Pb content just after 7 days of exposure ($p < 0.05$) and CUB animals also showed statistical differences, higher at T-7(0.065 ± 0.008) and lower at T-21 (0.031 ± 0.010).

DNA-SB (μg DNA/mg prot) used for genotoxicity indication,

Table 3
Percentage of Biologically Active Metals [P3, P4 and P5 = BAM] and Biologically Detoxified Metals [P2 and S5 = BDM] of Pb from the hepatopancreas and gills of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time T (0 [before the experiment], 7, 14, 21 and 28 days) exposed to lead concentration in water (10.6 ± 1.4 μg L^{-1}).

Exposure time (days)	Treatment	Hepatopancreas		Gills	
		BAM (%)	BDM (%)	BAM (%)	BDM (%)
T - 0	Cont/Jur	1.18	98.82	0.41	99.59
	Cub	0.52	99.48	0.58	99.42
T - 7	Cont	0.81	99.19	7.23	92.77
	Jur	0.63	99.37	0.41	99.59
	Cub	0.44	99.56	7.62	92.38
T - 14	Cont	0.12	99.88	0.23	99.77
	Jur	0.80	99.20	1.21	98.79
	Cub	1.31	98.69	12.01	87.99
T - 21	Cont	0.74	99.26	5.96	94.04
	Jur	1.56	98.44	4.49	95.51
	Cub	0.88	99.12	5.17	94.83
T - 28	Cont	1.81	98.19	0.60	99.40
	Jur	1.44	98.56	12.81	87.19
	Cub	10.37	89.63	18.56	81.44

demonstrated that JUR crabs (24.2 ± 1.4) differed statistically from CONT (13.8 ± 3.8) and CUB treatments (11.2 ± 8.4) during almost the entire experiment ($p < 0.05$), with exception of CUB at T-14. About the genotoxicity indicated by micronuclei (MN‰), CONT animals presented mean values of 1 ± 1 at T-28 to 1.8 ± 1.3 at T-0), differing statistically from the other two treatments in all exposure conditions ($p < 0.05$). JUR specimens presented the highest genotoxic damages observed after 14 days of exposure, reaching mean values of 17 micronuclei cells (±2.7 MN‰). Actually, crabs from this treatment showed higher quantities of micronuclei in all reading times, compared to before exposure (T-0) ($p < 0.05$). CUB animals always presented above 7 micronucleated cells per 1000 analyzed throughout the experiment, including before the exposure to the metal(T-0).

During Pb exposure, CONT crabs presented a neutral red retention time statistically higher (82.5 ± 2.1 min) than the animals from JUR (33 ± 9.4 min) and CUB (43.2 ± 8.1 min) during the entire experiment ($p < 0.05$). Higher cytoxicity observed in crabs belonging to JUR was observed during all reading times after lead exposure ($p < 0.05$). No statistical difference ($p > 0.05$) was recorded between JUR and CUB during the experiment.

3.4. Association of lead bioaccumulation (total and partitioned) and its sublethal effects

From the multivariate analysis, presented in Fig. 4, the original set of variables could be narrowed down to four new factors, which explained 81.3% of the total variance as presented in Table 5. The first principal factor (F1) accounted for 41.3% of the variance and highlighted the relationship between NRT, BAM (gills [G] and hepatopancreas [H]), BDM (H), DNA-SB, Pb Total and ALA-D, both in gills. The treatments associated to these effects were JUR and CUB at T-28. The second principal factor (F2) accounted for 17.4% of the variance and pointed the MN, NRT, BDM (in G and H), Pb Total (in H) and DNA-SB. To F2 the main groups linked with these results were also JUR (T-14) and CUB (T-14 and T-21). The third principal factor (F3) accounted for 10.6% of the variance and presenting relationship between the biomarkers MN and METALO (in G). A negative association was also recorded, the ALA-D (in H). The treatments linked to these positive and negative effects were JUR and CUB both after 7 days. The last principal factor (F4) accounted for 9.2% of the variance and showing association only to gills responses, specifically between BAM, ALAD and METALO. JUR and CUB were again related with gills responses after 21 and 28 days, respectively.

4. Discussion

The Pb exposure setup worked properly and kept the physicochemical conditions constant, without any significant changes. Under such conditions, *U. cordatus* individuals maintained their physiological status avoiding biased results (Christofoletti et al., 2013; Artal et al., 2018; Pinheiro et al. 2018). Similarly, size and sex of the studied specimens were standardized as suggested by Duarte et al. (2019).

Over 50% of lead emissions have been originated from petrol during the last century. The metal levels increased and polluted the natural environments (Jä;rup, 2003), especially in estuarine systems (Duarte et al., 2017; Kulkarni et al., 2018; Prasad et al., 2019). As a result, crab species have been intensely exposed to this contaminant, bio accumulating it and suffering several biological injuries (Ferrer et al., 2006; Macfarlane et al., 2006; Pan et al., 2011; Li et al., 2017; Xu et al., 2019).

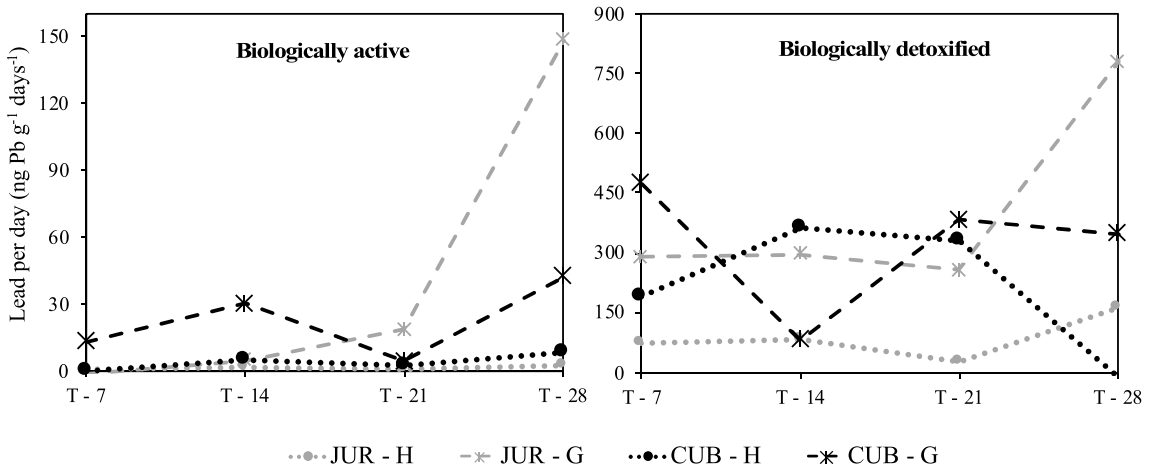

Fig. 3. Pb net accumulation rates (ng g-1 day-1) as Biologically Active [P3, P4 and P5 = BAM] (left) and Biologically Detoxified [P2 and S5 = BDM] (right) from the hepatopancreas (H) and gills (G) of *Ucides cordatus* by treatment (Juréia, JUR and Cubatão, CUB) and exposure time (7, 14, 21 and 28 days) exposed to lead concentration in water (10.6 ± 1.4 μg L^{-1}).

Table 4
Mean and standard deviation of DNA Strand Break (DNA SB μg DNA/mg prot), micronucleus frequency (MN/1000 cells), neutral red retention time (NRRT minutes), levels of enzymatic activity of aminolevulinic acid dehydratase (ALA-D in μg PBG/mg prot*h) and Metallothioneins (MTs) content in tissues (hemolymph, hepatopancreas and gill) of *Ucides cordatus* by treatment (Control, Juréia and Cubatão) and exposure time T (0 [before the experiment], 7, 14, 21 and 28 days) to lead concentration in water ($10.6 \pm 1.4\ \mu g\ L^{-1}$). 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 (μg DNA/mg prot)	MN (‰)	NRRT (min.)	ALA-D (μg PBG/mg prot*h)		METALO (mg MT/mg prot)	
		Hemolymph	Hemolymph	Hemolymph	Hepatopancreas	Gill	Hepatopancreas	Gill
T-0	Cont/Jur	13.2±2.5a	1.8±1.3a	85.5±7.2b	1.890±0.910a	0.269±0.170a	0.027±0.011a	0.038±0.004a
	Cub	12.5±0.7a	8.4±1.2b	48.0±9.5a	1.850±0.460a	0.180±0.100a	0.030±0.004a	0.041±0.008a
T-7	Cont	11.1±3.0a	1.6±1.1a	81.0±8.2b	1.796±0.431b	0.144±0.1857a	0.026±0.029a	0.038±0.014a
	Jur	22.1±5.5b	12.4±1.1b	42.0±6.7a	0.665±0.349a	0.367±0.2527a	0.032±0.010a	0.059±0.010ab
	Cub	22.9±4.1b	11.4±3.5b	48.0±6.7a	0.623±0.138a	0.323±0.1891a	0.030±0.005a	0.065±0.008b
T-14	Cont	13.8±3.8a	1.2±0.8a	81.0±8.2b	0.651±0.165a	0.127±0.0173a	0.023±0.002a	0.037±0.012a
	Jur	24.2±1.4b	17.0±2.7c	39.0±8.2a	0.571±0.311a	0.141±0.0681a	0.041±0.012a	0.031±0.010a
	Cub	15.3±6.4a	12.2±4.2b	51.0±8.2a	0.371±0.096a	0.061±0.0075a	0.032±0.003a	0.044±0.015a
T-21	Cont	12.6±3.2a	1.4±1.1a	81.0±8.2b	1.483±0.250a	0.075±0.0173a	0.023±0.008a	0.026±0.009a
	Jur	19.1±4.1b	10.7±2.2b	30.0±10.6a	1.964±0.708a	0.046±0.0681a	0.040±0.014a	0.048±0.008b
	Cub	26.7±2.1b	12.8±4.3b	36.0±8.2a	1.856±0.127a	0.054±0.0075a	0.033±0.003a	0.031±0.010ab
T-28	Cont	13.0±1.8a	1.0±1.0a	84.0±8.2b	1.4051±0.146a	0.170±0.0909a	0.025±0.007a	0.039±0.011a
	Jur	28.5±4.1b	10.4±2.9b	21.0±8.2a	1.1709±0.297a	0.276±0.1646a	0.027±0.013a	0.037±0.003a
	Cub	20.8±1.5b	7.±2.7b	33.0±12.5a	1.0852±0.534a	0.258±0.2905a	0.061±0.011b	0.047±0.012a

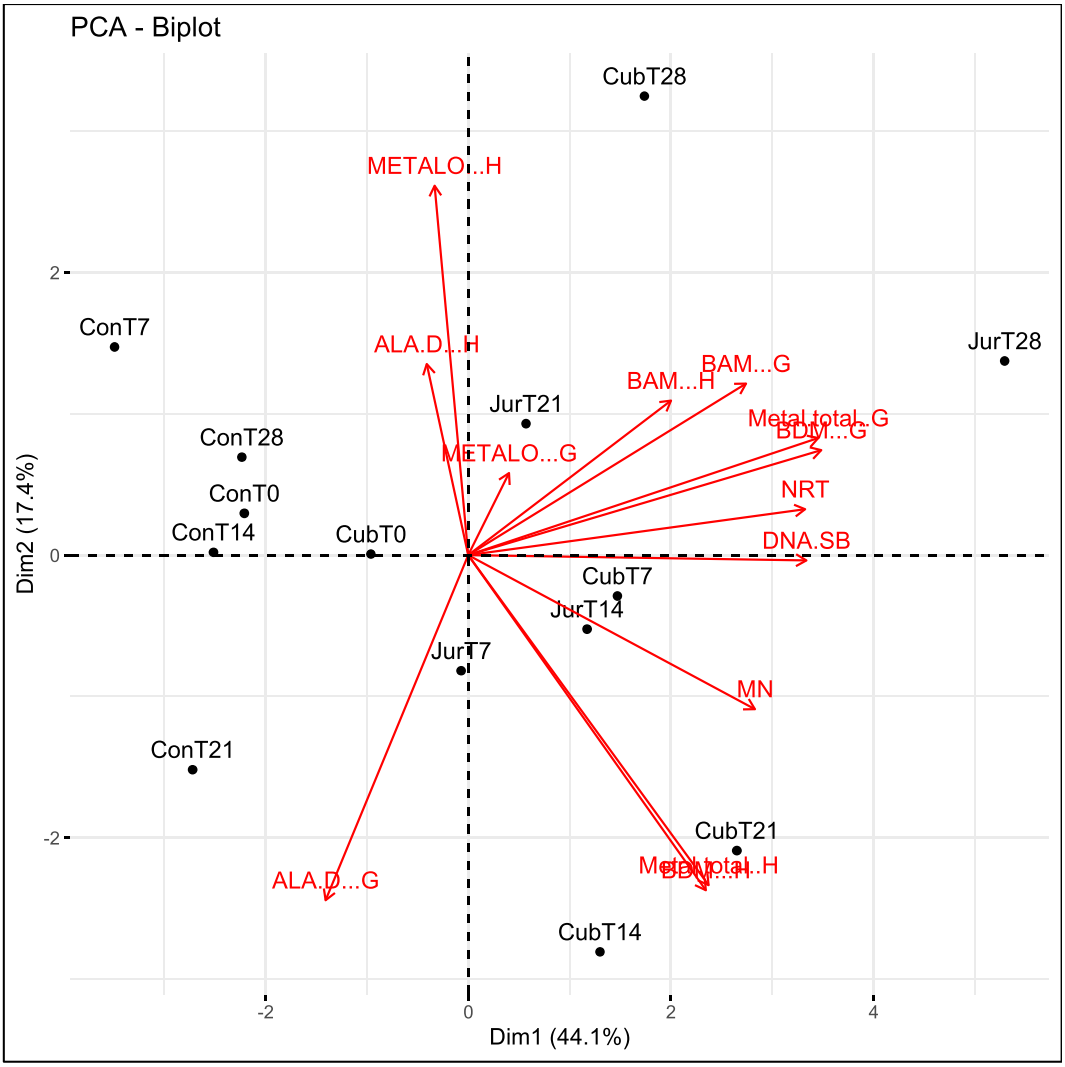

Fig. 4. Principal Component Analysis (PCA) using biplot function, namely: similarity distances between dependent variables (Biologically Active Metals [BAM] and Biologically Detoxified Metals [BDM] of Pb, levels of enzymatic activity of aminolevulinic acid dehydratase [ALA-D], DNA Strand Break [DNA-SB], micronucleus frequency [MN], Metallothioneins (METALO) content, neutral red retention time, NRRT], from tissues [hemolymph, hepatopancreas - H and gill - G]), and independent variables (treatments [Control, Con; Juréia, Jur; Cubatão, Cub] and exposure times [before, T-0; after, T-28]) recorded for *Ucides cordatus* species exposed to lead in water at a mean concentration of $10.6 \pm 1.4\ \mu g\ L^{-1}$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 5
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
	44,1	17,4	10,6	9,2
MN		0,79	0,44	
NRT	0,56	0,63		
BAM - H	0,40			0,48
BDM - H		0,90		
Metal total -H		0,90		
BAM - G	0,91			
BDM - G	0,88	0,41		
Metal total- G	0,90			
ALA-D - H			−0,58	0,56
ALA-D - G	−0,74			
METALO - H				0,92
METALO - G			0,78	
DNA-SB	0,61	0,60		

In this investigation, *U. cordatus* individuals were exposed for a long term (28 days) to a Pb concentration which was supposedly non-threatening (10.6 ± 1.4 μg L^{-1}) if the Brazilian legislation is taken into account (Conama 357/2005; Church et al., 2017). This Pb level has been observed in São Paulo State estuaries and hence is of relevance for environmental status studies (Pinheiro et al. 2012, 2013; Duarte et al. 2017).

At both treatments (JUR and CUB), the gill was the preferential organ for Pb accumulation after the 28-day exposure time. This same pattern was also observed to coastal crab species, for example, the green crab *Carcinus aestuarii* (Selvi et al., 2012), blue crabs *Callinectes sapidus* (Çoğun et al., 2017), *Callinectes danae* (Bordon et al., 2018) and root crab *Goniopsis cruentata* (Costa et al., 2018). Peng et al. (2011) observed similar concentrations of Pb across the different tissues of the hydrothermal vent crab *Xenograpsus testudinatus* (carapace, gills, musculature and hepatopancreas).

Besides gills, the carapace and gonad tissues were important compartments for lead bioaccumulation in crabs exposed to lead. Du Preez et al. (1993) studying the dosage of this metal in several tissues of the freshwater river crab, *Potamonautes warreni,* also found relevant values in carapace and gonads. Specifically to carapace, crustaceans have this structure formed primarily for calcium carbonate (Luquet and Marin, 2004) and the calcium ions (Ca^{2+}) are transported across the membrane by carriers in well-defined systems which require energy for ion uptake gradients, such as Na^{+} K^{+}ATPase (Lucu and Towle, 2003; Freire et al., 2008). Lead is, therefore, incorporated into these systems, particularly if they are in ionic size similar to the electrolyte that is being transported. The Pb ion has an ionic radius (1.19 Å) similar to calcium (0.99 Å) and is capable of entering into the cell using the same calcium channels (Rainbow and Black, 2005; Rainbow, 2007). However, there is a beneficial aspect to crabs about it. Bergey and Weis (2007) described that molting is a fundamental pathway for Pb depuration in crustaceans, for example, the fiddler crab, *U. pugnax*, can reach 76% of lead elimination in contaminated mangrove. In relation to the bioaccumulation also highlighted in the gonads, these tissues are rich in lipids and lead, being lipophilic, presents high chemical affinity with them (Ayed, 2011). This also explains the high relative concentration of Pb observed in crab gonads exposed to the metal (JUR and CUB).

After 28 days of exposure, Juréia crabs accumulated statistically more Pb in four of the six quantified tissues (gills, carapace, gonads and musculature) if compared to Cubatão crabs. This result suggests that crabs from pristine mangroves have physiologically more difficulty to excrete Pb, since they have nearly twice as much of its bioaccumulation (Total sum in tissues: CUB = 10.12 ng g^{-1} and JUR = 18.77 ng g^{-1}).

The Juréia crab vulnerability supposition was confirmed by the obtained Pb accumulation rates, as animal gills from this treatment uptook more lead in the biologically active (BAM: 3.5 times higher) and detoxified (BDM: 2.2 times higher) compartments after 28 days of exposure than Cubatão crabs. Although not significantly, CUB crabs could allocate more Pb (BAM and BDM) to the hepatopancreas, the main detoxifying tissue. A possible evidence of adaptation and resistance to the metal (Rainbow, 2007; Eisler, 2010).

U. cordatus stored Pb in detoxified form predominantly (overall mean of 98.4% to hepatopancreas and 94.5% to gills). This is remarkable since lower values were observed for other animals such as the bivalve mollusk *Anadara trapezia* (66% detoxified in gills and 49% in hepatopancreas) (Taylor and Maher, 2012). In the case of Cd, for *Crassotrea gigas*, 45% was detoxified in hepatopancreas while 60% was in gills (Cao et al., 2018) while in fiddler crab, *Uca pugnax*, less than 40% was observed to the same tissue. (Khoury et al., 2008). Cd detoxification with very similar results (97.7% to hepatopancreas and 95.8% to gills) was observed for the same species (Duarte et al., 2019). When compared to those in the present study. These authors also observed subcellular fraction of this metal more in granules and heat stable metallothionein-like proteins (both gills and hepatopancreas) and this result is in agreement with the literature (Taylor and Maher, 2012; Rosabal et al., 2015; Campana et al., 2015). However, Piola and Johnston (2006a, 2006b) alert about the extra metabolic cost of the physiological handling of accumulative storage in detoxified form, is more than offset by the fact that its absence means non-survival.

Lead toxic injuries in cells involve several mechanisms (Li et al. 2015). It usually occurs by replacing Zn and Fe (divalent ions) and by the mimicry of calcium (Ballatori, 2002). For this reason, Pb was recorded in high concentrations in carapace of exposed crabs. Pb exposure could drive cell damage from oxidative stress (Meng et al., 2018), which is determined by an imbalance between the biological antioxidant defense system production and reactive oxygen species (Li et al. 2015). These sublethal injuries were described by Li et al. (2016) in sperm of the crab *Sinopotamon henanense* which presented a decline in its quality and by Lavradas et al. (2014) who also recorded oxidative stress to blue crab (*Callinectes* sp.) during Pb exposure.

To assess the lead toxicity at supposedly safe concentration in water (Conama 357/2005; Church et al., 2017), the present study used the results of multiple biomarkers. The ALA-D enzyme is usually inhibited as a consequence of exposure to lead (Suzen et al., 2003; Alves Costa et al., 2007; Kalman et al., 2008; Fernández et al., 2015; Kayaalti et al., 2016), however, the concentration used in this investigation was not high enough to cause inhibition of its activity neither in hepatopancreas nor in gills.

Metallothioneins (METALO) are usually considered appropriate biomarkers to indicate metal contamination (Viarengo et al., 1999; Monserrat et al., 2007; Yang et al., 2019). These low molecular weight proteins have the capacity to immobilize metals including in crustaceans (Ahearn et al., 2004; Ruttkay-Nedecky et al., 2013). Ortega et al. (2016) and Duarte et al. (2019), also studying U. cordatus species. observed that crabs were not producing metallothioneins proportionally (dose-response) to Cd concentration. About Pb, Bordon et al. (2018) studying its bioaccumulation and metallothionein levels to the crab *Callinectes danae* in relation to dietary and waterborne exposures, evidenced that both pathway exposures acted simultaneously to induce METALO increase (reaching a mean of 40 μg/mg), however, the specimens were

exposed to higher Pb concentrations (0.5 and 2.0 μg/g). Matin et al. (2019) found the same pattern of METALO induced by Pb in the hermit crab *Clibanarius signatus* and concluded that this technic could be useful as a first indication of metal pollution in coastal areas. Similarly, in the present study, even in low Pb concentrations, hepatopancreas of the crabs exposed to Pb triggered their defense mechanisms expressing more METALO after 7 and 21 days. Gills of animals from Cubatão mangrove demonstrated such METALO increase after 28 days, recording values more than twice the observed in Juréia crabs.

DNA damage may determine mutations, altered bases, strand breaks (Shugart, 2000), teratogenesis and carcinogenesis (Järup, 2003; Fouani et al., 2017). Genotoxicity could imply in genetic erosion and threaten the species resilience face to environment pressures (Monserrat et al., 2007; Bijlsma and Loeschcke, 2012), causing damage to populations (Duarte et al., 2016) and hazards to communities (Pereira et al., 2011, 2014). Fossi et al. (2000) documented genotoxicity in the crab *Carcinus aestuarii* due to polycyclic aromatic hydrocarbons (PAHs). About lead, Duarte et al. (2017) evidenced a significant association between this metal and genotoxic impact recorded in *U. cordatus*. In the present study, even supposedly in safe concentration of Pb, exposed crabs (JUR and CUB) showed higher DNA damage (MN and DNA-SB) during the experiment.

Cytotoxic injuries occur by lipoperoxidation of cell membranes, resulting in integrity and permeability disturbance (Svendsen et al., 2004). Buratti et al. (2012) showed a strong correlation between membrane stability of haemocytes lysosomes of the crab *Carcinus maenas* and metals contamination in sediments from Algeciras Bay, Spain. In a "*in situ*" study integrating chemical and biomarkers analyses, Duarte et al. (2017) presented an estimation of cytotoxic impacts related to lead concentration in abiotic compartments (water: 50% probability [CC50] = 0.171 m L^{-1}; sediment: cytotoxic probability [CC50] = 6.49 μg/g). However, the data showed here evidenced toxicity in a much lower level in water.

Cyto-genetic damage responses in control group were statistically different from the crabs exposed to lead and it is necessary to consider the relevance of such distress., Duarte et al. (2016) suggested damage categories for mangrove areas (PNI = probably no impact, PLI = probable low impact, and PHI = probable high impact) based on genotoxicity (MN‰) and cytotoxicity (NRRT) responses for *U. cordatus*. Crabs exposed to Pb in concentration considered safe showed probable high impact in 100% of the cases. Multivariate analyzes confirmed the associations between Pb bioaccumulation (active, detoxified and total) and sublethal genocytotoxic damages observed in exposed crabs, corroborating the Pb toxicity even at a supposedly safe concentration. Such alterations at sublethal levels are recognized as indicators of prepathological issues (Pereira et al., 2014; Duarte et al. 2016) and are of large ecological relevance (Amiard-Triquet et al., 2013), since they can display cellular death, leading to cascading damage impacts to up population-community level (Choueri et al. 2009; Duarte et al. 2016). Pereira et al. (2014), for example, recorded a significant association between these biomarkers and some ecological indexes. Thus, geno-cytotoxicity can indicate threat to tropical estuarine systems (Pinheiro et al. 2012; Duarte et al. 2016). It is worth note the Pb water concentration used in the present experiment was previously documented at São Paulo coastal zone mangroves (Pinheiro et al. 2012, 2013; Duarte et al. 2017).

Tolerance could be related to physiological acclimatization (that occurs during the organism's life cycle) or to genetic selection of the population by long-term selective pressure (e.g., by epigenetic processes and molecular mechanisms, see Espinosa et al., 2007; Zhao et al., 2015; Topal et al., 2017). Lead toxicity was assessed on a sentinel species subpopulation exposed to different conservation status mangroves and it is interesting to clarify whether the crabs from the polluted area (CUB) seemed to present a higher tolerance to Pb, similarly to the results observed by Duarte et al. (2019) for cadmium. In the present study, results suggest that animals from CUB acquired biological tolerance. Crabs from pristine area exhibited higher Pb accumulation (almost twice in total and more in four of the six quantified tissues); uptook more biologically active forms of Pb after 28 days (BAM: 3.5 times higher) and presented higher genotoxic damage (MN and DNA-SB) after 14 days of exposure. Thus, animals from pristine locations, such as protected areas, could be less capable to resist to lead pollution, which alert about the need of greater conservation effort.

The employed approach could assess the toxicity of other metals in water concentrations. Our results indicated that supposedly Pb safe doses are capable of causing relevant damage effects on sentinel species. Thus, further studies should assess no-effect metal concentrations in order to adjust the laws towards the effective conservation of tropical estuaries.

5. Conclusions

The species *U. cordatus* is a good biomonitor for Pb exposure, and gills are the target tissues. The organism allocated Pb mainly in the detoxified form. However, even in supposedly safe dosage, crabs triggered their defense mechanisms, producing more metallothioneins and presenting relevant cytogenotoxic damage. Moreover, crabs from the polluted mangrove seemed to exhibit biological tolerance to lead. The results provided a new view about Pb toxicity even in concentration considered environmentally safe, which could support the management of estuarine areas taking into account differential conservation status.

Credit author statement

Luis Felipe de Almeida DUARTE (*Corresponding author*) = Conducting the entire investigation since the beginning. Conceptualization, development of methodology, application of statistical, performing the experiment and all biomarkers. Preparation, creation and presentation of the manuscript.

Julián BLASCO = Acquisition of the financial support for the project leading to this publication, provision of his laboratory in Spain, management and coordination responsibility for the research activity planning (about Pb subcellular partition and biomarkers' responses [metallothioneins, enzymatic activity of aminolevulinic acid dehydratase]), provision of study materials (reagents and materials), and writing of the paper.

Marília Gabriela Miranda CATHARINO = provision of study materials (reagents and materials to bioaccumulation), quantification of total bioaccumulation in six tissues of *U. cordatus* crabs and writing of the paper.

Edson Gonçalves MOREIRA = translate of the paper to English language, provision of study materials (reagents and materials to bioaccumulation), quantification of total bioaccumulation in six tissues of *U. cordatus* crabs and writing of the paper.

Chiara TROMBINI = responsible for metallothioneins and enzymatic activity of aminolevulinic acid dehydratase analysis and writing of the paper.

Caio Rodrigues NOBRE = development of methodology, performing the experiment and some biomarkers (DNA strand breaks, micronucleated cells and neutral red retention time). Help in writing of the paper as well.

Beatriz Barbosa MORENO = development of methodology, performing the experiment and some biomarkers (DNA strand

breaks, micronucleated cells and neutral red retention time). Help in writing of the paper as well.

Denis Moledo de Souza ABESSA = provision of study materials (reagents and materials to DNA strand breaks analysis), provision of his laboratory in Brazil (UNESP), application of statistical, biomarker DNA strand breaks and writing of the paper as well.

Camilo Dias Seabra PEREIRA = Conceptualization, development of methodology, application of statistical, acquisition of the financial support in partnership with Duarte, provision of his laboratory in Brazil (UNIFESP), management and coordination responsibility for the research activity planning and writing of the paper as well.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126394.

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